MOLECULAR GENETICS OF THE ANGELMAN SYNDROME

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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This dissertation is dedicated to
C. Andrew Glomski
for his unending belief, encouragement and support

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MOLECULAR GENETICS OF THE ANGELMAN SYNDROME

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Genomic imprinting is a parent-of-origin epigenetic phenomenon that is intimately involved in development, cancer and several genetic disorders, including Angelman syndrome (AS). AS is a severe neurobehavioral disorder characterized by four cardinal features: 1) severe developmental delay; 2) profound speech impairment; 3) a characteristic movement disorder; and 4) an AS-specific behavior, which includes an inappropriately happy affect. My studies revealed that AS is caused by 5 molecular mechanisms: Class I) 4 Mb deletions; Class II) paternal UPD; Class III) imprinting defects; Class IV) intragenic mutations in *UBE3A*, the E3 ubiquitin protein ligase 3A gene; and Class V) no known defects. Our statistical models demonstrated that several significant phenotypic differences occur among the five molecular classes. Patients can be separated into four phenotypic groups: Class I, Classes II and III, Class IV, and Class V. Class I patients are the most severely affected, while Classes II and III

are the least. Class I patients comprise a contiguous gene disorder, with haploinsufficiency of multiple genes exacerbating the lack of the maternal *UBE3A* gene. Multiple clinical distinctions can be made between the classes, with differences most striking in pigmentation; growth parameters; achievement of developmental milestones; and severity, frequency and age at onset of seizures.

Initial experiments showed that imprinted expression of *UBE3A* was limited to the brain in humans, and revealed the presence of a brain-specific antisense transcript that overlapped the 3' end of *UBE3A*. In our studies, RT-PCR analyses of *UBE3A* and its antisense transcript demonstrated discordant imprinted expression of the two genes in several regions of the human brain. *UBE3A* was preferentially expressed from the maternal allele, while the antisense transcript was preferentially expressed from the paternal allele. In addition, DNA methylation analysis revealed that 5' *UBE3A* does not demonstrate a DNA methylation imprint. All tissues, even those that demonstrated imprinted expression of *UBE3A*, are unmethylated in the promoter region of the gene. The lack of a DNA methylation imprint at *UBE3A* and the observation of discordant imprinted expression of the two genes support a role for the antisense transcript in the regulation of imprinted expression of *UBE3A*.

INTRODUCTION

Genomic Imprinting Is an Epigenetic Phenomenon

Epigenetic phenomena are implicated in several diseases, cancer, X chromosome inactivation and genomic imprinting. In addition, epigenetic events such as DNA methylation and histone acetylation play major roles in the regulation of gene expression. Epigenetic effects occur when the primary genetic material is heritably altered in somatic and/or germ cells, causing changes in expression, without the creation of a mutational event in the DNA.

One of the most familiar epigenetic processes is genomic imprinting, which occurs when one allele of an imprinted gene is transcriptionally activated or silenced in a parent-of-origin dependent manner. The parental origin of the allele determines whether an imprinted gene will be expressed; some imprinted genes are only expressed from the paternal allele, while others are expressed exclusively from the maternal copy.

Two of the most extreme examples of genomic imprinting are the complete hydatidiform mole and ovarian teratoma (Kajii et al., 1984; Ohama et al., 1981; Ohama et al., 1985). Hydatidaform moles occur when an anuclear egg is fertilized by either one or two haploid sperm, with duplication of the entire haploid genome occurring when a single sperm fertilizes the egg. Hydatidaform moles, which are completely androgenetic (Ag), give rise to hyperplastic extraembryonic growth with little or no fetal development. *In situ* activation and duplication of the maternal DNA

of an unfertilized oocyte produces an ovarian teratoma, which is completely parthenogenetic (Pg), having two complete copies of the maternal genome. Although ovarian teratomas have very little extraembryonic development, they can develop into a disorganized but well-differentiated tumor that often reveals remnants of mesenchymal and endodermal structures.

Elegant experiments in the 1980s revealed the consequences of uniparental mammalian development in the mouse. Pronuclear transfer experiments showed that androgenetic and parthenogenetic embryos failed to develop to term (Barton et al., 1984; McGrath and Solter, 1984). These mice closely resembled their human counterparts, complete hydatidaform moles and ovarian teratomas, and demonstrated that the paternal germ line was required for differentiation of extraembryonic tissues, while the maternal genome was necessary for embryonic development.

Subsequent examination of mice with uniparental disomy (UPD) for various chromosomal regions revealed the presence of 10 imprinted domains on seven mouse chromosomes: 2, 6, 7, 11, 12, 17 and the X (John and Surani, 1996). In humans, UPD of chromosomes 2, 7, 11, 14, 15, 16, and 20 has also been shown to cause aberrant phenotypes (Ledbetter and Engel, 1995). To date, over 35 imprinted genes and 21 imprinted syndromes have been identified in human and mouse (Falls et al., 1999; Nicholls, 2000). These genes are involved in fetal and placental growth, cell proliferation and neurobehavior. Two large clusters of imprinted genes reside on human chromosomes 11p15.5 and 15q11-13. Genes in the Beckwith-Wiedemann syndrome region on 11p15.5 have been shown to be involved in fetal growth and cell proliferation, while the Angelman/Prader-Willi syndrome (AS/PWS) region on 15q11-13 contains imprinted genes that influence neurobehavior. A review of these two systems follows.

Genomic Imprinting and Beckwith-Wiedemann Syndrome

Clinical Features of Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome (BWS) is a multi-faceted genetic disorder characterized by fetal and neonatal overgrowth, macroglossia and abdominal wall defects. Additional common features include hemihypertrophy, genitourinary abnormalities, organomegaly and Wilm's tumor (Maher and Reik, 2000). Three major types of BWS patients have been identified: patients with chromosome anomalies, sporadic cases or familial inheritance. The finding of a significant percentage of sporadic patients with partial paternal UPD for chromosome 11p and the presence of duplications, inversions and breakpoints of balanced translocations have localized the genes for BWS to 11p15.5 (Maher and Reik, 2000). Ten imprinted genes have been mapped to this region, which is flanked by non-imprinted genes, and at least two genes within the cluster demonstrate biallelic expression, subdividing the cluster into two imprinted domains (Figure 1-1).

Imprinted Genes in BWS

The *CDKN1C*, *KvLQT1*, *IGF2* and *H19* genes from 11p15.5 have been the most studied, and it appears that both of these groups of imprinted genes are involved in the pathogenesis of BWS. Recent experiments suggest that these two regions are under the control of two distinct imprinting centers (Lee et al., 1999b; Reik et al., 1995; Smilinich et al., 1999). The majority of sporadic BWS patients show loss of imprinting (LOI) at *IGF2*, identified by the activation of both parental alleles (Maher and Reik, 2000). Two mechanisms are associated with LOI at *IGF2*. In the first, LOI at *IGF2* was accompanied by a change in the epigenotype of *H19*, which silenced the maternal *H19* allele (Reik et al., 1995). In the second, the *H19* epigenotype was

normal, but the DNA methylation imprint on the maternal allele of *KvLQT1* became unmethylated, inducing expression of the *KvLQT1* antisense transcript, *LIT1* (Brown et al., 1996; Smilinich et al., 1999). Therefore, these observations show that disruption of the epigenotype at either *H19* or *KvLQT1* can cause LOI of *IGF2* in patients with BWS.

Imprinting Mechanisms of *Igf2* and *H19*

An elegant series of experiments by several groups has helped to tease out the important elements of imprinted expression of *Igf2* and *H19* in the mouse (reviewed in Brannan and Bartolomei, 1999). Subsequent experiments have revealed the mechanism of imprinted expression of these two genes (Figure 1-2; Bell and Felsenfeld, 2000; Drewell et al., 2000; Hark et al., 2000; Ishihara et al., 2000; Szabo et al., 2000). These latter experiments revealed that the differentially methylated region located at 5' *H19* was a boundary element that when unmethylated, bound a repressor protein, preventing the *Igf2* gene from being activated by upstream enhancers.

The identification of the mechanism for imprinted expression of *Igf2*/*H19* is a first for imprinted genes. It will be interesting to discover how mutations in upstream genes can also disrupt imprinting of *Igf2*. Perhaps there are two levels of control in this region: local and regional, with long-range interactions occurring between *KvLQT1* and *IGF2*. It will also be interesting to determine what phenotypic effects other imprinted genes in this region have on subsets of BWS patients.

The Angelman and Prader-Willi Syndromes

Research over the last five years has yielded many clues as to the factors necessary for genomic imprinting of the Angelman (AS) and Prader-Willi (PWS)

syndromes. Novel imprinted genes have been identified on 15q11-13, mutations in the E6 ubiquitin protein-ligase gene, *UBE3A*, were found in several patients with AS, new methods of transmission of AS and PWS were identified, and studies of patients with imprinting defects revealed that the imprinting center consisted of separate AS and PWS components. In addition, multiple mouse models of AS and PWS were created and null mutations of several imprinted genes were generated in the mouse.

Clinical Features of AS and PWS

The Angelman and Prader-Willi syndromes delineate the second major cluster of imprinted genes in the human. Although both syndromes are associated with 4 Mb interstitial deletions of chromosome 15q11-13, AS and PWS are very different clinical neurobehavioral disorders. AS is characterized by severe mental retardation, profound speech impairment, a movement and balance disorder and an AS-specific behavior which includes an inappropriately happy affect interspersed with paroxysms of laughter (Williams et al., 1995a; Williams et al., 2000). In stark contrast, PWS is initially characterized by neonatal hypotonia and failure to thrive. However at 18 to 36 months of age, patients become hyperphagic, eventually leading to morbid obesity. They also have mild mental retardation, hypogonadism, small hands and feet and a characteristic behavior which includes lethargy, aggressiveness and obsessive-compulsive disorder (Holm et al., 1993).

The differential phenotype depends upon the parental transmission of the 15q11-13 defect with normal development requiring biparental genetic contribution of 15q11-13. AS is caused by a lack of maternal contribution, while lack of paternal contribution from 15q11-13 results in PWS.

Molecular Mechanisms of AS and PWS

The genetics of the AS and PWS syndromes are very complex; multiple different chromosome 15 defects occur in these two distinct syndromes (Figure 1-3). Loss or inactivation of at least one 15q11-13 gene occurs in both the AS and PWS syndromes, with maternal deficiencies giving rise to AS children and paternal deficits causing PWS (Figure 1-3). Five major molecular classes of AS patients have been identified: I) 4 Mb maternal deletions of 15q11-13; II) paternal uniparental disomies (UPD); III) imprinting defects (ID); IV) patients with mutations in a single gene, *UBE3A* and V) patients with no known defects. Prader-Willi patients have been identified who belong in the first three classes: paternal deletions of 15q11-13, maternal UPDs and ID. No single gene mutations have been found in PWS patients, suggesting that PWS is part of a contiguous gene syndrome with inactivation of at least two genes necessary for the full phenotype to develop.

Genes Involved in AS and PWS

This past year has been exciting in the AS/PWS field. Nine novel imprinted genes and transcripts were localized to the 15q11-13 imprinted domain (Boccaccio et al., 1999; Farber et al., 2000; Lee et al., 2000; Lee and Wevrick, 2000), with 18 paternally expressed transcripts having been mapped to the AS/PWS imprinted domain to date: cen-*MKRN3*, *MKRN3-AS*, W1-15987, *NDN*, *MAGEL2*, *IC* transcript, *SNURF-SNRPN*, *PAR5*, W1-15028, W1-13791, *IPW*, *PAR1*, stSG12920, stSG3346/stsN21972, BCD1279, A005C48 and *UBE3A-AS*-tel. Although no sequence mutations have been found in any of these genes in PWS patients, expression studies indicate that PWS patients do not express *MKRN3*, *MKRN3-AS*, W1-15987, *NDN*, *MAGEL2*, *IC* transcript, *SNURF-SNRPN*, *PAR5*, W1-15028, W1-13791, *PAR1*, stSG12920, stSG3346/stsN21972, or A005C48 in lymphoblasts, skin fibroblasts or human brain (Figure 1-4A, B).

Interestingly, *IPW* demonstrates imprinted expression in lymphoblast and skin fibroblast cell lines, but biallelic expression in human brain. All of these genes and transcripts could potentially be involved in the etiology of PWS.

Only one gene in the AS/PWS imprinted domain, *UBE3A*, demonstrates a maternal-only expression imprint (Rougeulle et al., 1997; Vu and Hoffman, 1997). However, unlike most of the paternally expressed 15q11-13 genes, imprinted expression of *UBE3A* is restricted to brain (Figure 1-4A, B). Mouse studies revealed that tissue-specific imprinted expression of *Ube3a* was restricted to the Purkinje cells of the cerebellum, the CA2 and CA3 regions of the hippocampus and the mitral cell layer of the olfactory bulb (Albrecht et al., 1997; Sutcliffe et al., 1996). In the human, much less is known about the tissue specificity of imprinted expression of *UBE3A*. All studies in the adult have relied on RT-PCR analysis from a single frontal cortex sample, and have shown that *UBE3A* imprinted expression varies within this tissue (Lee and Wevrick, 2000; Rougeulle et al., 1997). Research on human fetal brain demonstrated biased expression, but could not determine if the preferentially expressed allele was maternal or paternal in origin (Vu and Hoffman, 1997).

Mouse Models of PWS

Mouse models of all the molecular classes of PWS (deletions, UPD and imprinting defects) have been created. Two mouse models have been generated with large 7C deletions. One deletion, which occurred serendipitously by insertional mutagenesis, resulted in a mouse with a transgene-induced deletion of the entire orthologous region (Gabriel et al., 1999). When the $Tg^{PWS/AS(del)}$ was passed through the paternal germline, transgenic progeny showed neonatal lethality, and failed to express several PWS candidate genes, including *Snurf*, *Snrpn* and *Ndn*. The second deletion model was caused by a targeted disruption that extended from exon 2 of *Snurf/Snrpn*

to exon 8 of *Ube3a* (Tsai et al., 1999b). Paternal transmission of this deletion resulted in a less severe phenotype than the previous model; 50% of neonates survived past 12 days, and 20% survived past weaning. Although smaller than their wild type littermates, surviving deletion mice (male and female) were fertile, unlike most individuals with PWS.

A mouse model of PWS patients with UPD has also been generated. These mice, which had maternal UPD for mouse chromosome 7C, were the first murine model to recapitulate features of PWS (Cattanach et al., 1992). Similar to infants with PWS, these mice exhibited severe failure-to-thrive. Untreated, the mice died within seven days of birth. Expression analysis revealed a complete absence of *Snrpn* in these mice.

Targeted disruption of the PWS imprinting center (PWS-IC) has produced a mouse model of imprinting defects (ID; Yang et al., 1998). Paternal inheritance of this 42 kb IC deletion resulted in a mouse with a phenotype characterized by neonatal lethality and lack of expression of several paternal-only expressed genes within chromosome 7C. This important mouse model demonstrated that the PWS-IC was functionally conserved between mouse and human and provided a valuable model to study the mechanisms of imprinting in the mouse.

In addition, several studies have reported on the effects of disruption of three paternal-only expressed genes within 7C. An intragenic deletion of *Snrpn* (Yang et al., 1998) or a disruption of exon 2 of *Snurf* (Tsai et al., 1999b) produced normal mice, indicating that disruption of either *Snurf* or *Snrpn* was not sufficient to cause features of PWS. Targeted deletion of *Ndn* has resulted in two different outcomes. In one study, mice homozygous for a null mutation in *Ndn* were fully viable and did not develop any features common to PWS, including hypogonadism, infertility or obesity

(Tsai et al., 1999a). However in a second study, mice that inherited a mutated *Ndn* allele on the paternally inherited chromosome demonstrated early post-natal lethality (Gerard et al., 1999). These authors believe that the genetic background influenced the lethal phenotype.

Mouse Models of AS

To date, four mouse models of three Classes of AS (deletions, UPD and *Ube3a* mutations) have been created. In addition, an alternative AS model was also created from mice deficient in GABAß3. A deletion mouse was generated by maternal transmission of the $Tg^{PWS/AS(del)}$ transgenic mouse (Gabriel et al., 1999). Initial experiments revealed that this mouse model demonstrated imprinted expression of *Ube3a* in the cerebellum. A second deletion mouse has also been identified (Johnson et al., 1995). This mouse model exhibited a radiation-induced deletion that extended from *Ube3a* to the *p* locus. Mice with paternal UPD for mouse chromosome 7C demonstrated AS-like features including an ataxic gait, EEG abnormalities, reduced brain weight and late onset obesity (Cattanach et al., 1997). Targeted disruption of the maternal allele of *Ube3a* resulted in mice that were very similar to the UPD model, with poor motor skills, inducible seizures and context-dependent learning problems (Jiang et al., 1998). Mice homozygous for a null mutation in GABAß3 also displayed several features of AS (DeLorey et al., 1998), although heterozygotes were not statistically different from wild type littermates. These mice had several features common to individuals with AS including: abnormal EEG patterns, seizures, learning and memory deficits, poor motor skills, hyperactivity and a disturbed rest-activity cycle.

DNA Methylation: An Epigenetic Mark

DNA methylation analysis, first proposed by Driscoll and colleagues (1992), is now widely used by researchers and diagnostic laboratories to detect parental contributions based on the differential DNA methylation imprints associated with imprinted genes. Several sites in 15q11-13 including the *MKRN3* locus (Driscoll et al., 1992), *NDN* (Jay et al., 1997), intron 7 of *SNRPN* (Glenn et al., 1993b) and the CpG island of 5' *SNRPN* (Glenn et al., 1996; Kubota et al., 1996b; Sutcliffe et al., 1994) display parent-specific DNA methylation imprints in peripheral blood leukocytes and other tissues (Figure 1-4).

These imprints have been utilized to reliably diagnose AS and PWS patients who have deletions, UPD and ID (Clayton-Smith et al., 1993; Driscoll et al., 1992; Glenn et al., 1993a; Glenn et al., 1996). DNA methylation analysis of AS and PWS patients has led to the discovery of patients with defects in the imprinting process (ID). ID patients have biparental inheritance of chromosome 15 with no evidence of the common 4 Mb interstitial deletion, but still show uniparental DNA methylation imprints at loci extending over a 2 Mb region of 15q11-13 (Glenn et al., 1993a). About 50% of all AS and PWS patients with imprinting defects have microdeletions within the imprinting center (IC), which is located at the 5' end of *SNRPN* on 15q11-13 (Nicholls et al., 1998). The remaining ID patients have no detectable mutations, and are thought to have defects in the process of erasing and/or resetting the epigenotype in the germ line or somatic tissues (Buiting et al., 1998).

Structure and Function of the AS/PWS Imprinting Center

During each generation, the parental epigenetic mark must be erased in the germ line and reset according to the sex of the offspring, ensuring that each successive generation will receive the correct parental imprint in germ cells and

somatic tissue. Mistakes in the imprinting process would be detrimental, causing disease in subsequent generations based upon parental inheritance (Figure 1-5). Imprinting defects are responsible for 3-5% of all AS and PWS patients and disable the epigenetic switch from male to female or female to male, respectively. These deletions range from 6 to 200 kb and can regulate DNA methylation and expression imprints of genes located at least 1 Mb from the IC.

The identification AS and PWS individuals with imprinting defects launched a new field of research in an attempt to further understand the mechanisms of imprinted expression within 15q11-13 (Glenn et al., 1993a; Reis et al., 1994). Delineation of the deletion breakpoints in several ID patients localized the critical region for the IC to the promoter proximal region of *SNRPN* (Figure 1-4A; Buiting et al., 1995; Saitoh et al., 1997; Saitoh et al., 1996). The IC extends 150 kb centromeric to the *SNRPN* gene and encodes a transcript that contains alternatively spliced upstream exons of *SNRPN* (Dittrich et al., 1996). Although the *IC* transcript is not abundantly expressed, it demonstrates tissue-specific paternal-only expression, and is thought to be involved in the establishment of imprinted expression along 15q11-13.

Analysis of Class III AS and PWS individuals with deletions in the IC revealed that the imprinting center functions as a bipartite structure. Characterization of these deletion breakpoints narrowed the smallest region of deletion overlap (SRO) for the AS and PWS regions of the imprinting center (Figure 1-4A) to 0.9 and 4.3 kb, respectively (Buiting et al., 1999). All AS patients with IC mutations delete exon 5 of the *IC* transcript, while all PWS patients with IC mutations have deletions that extend into exon 1 of *SNRPN*.

UBE3A and the Ubiquitination Pathway

The involvement of *UBE3A* in the etiology of AS is now very well documented. Several laboratories have demonstrated protein truncating mutations of *UBE3A* in multiple AS patients (Baumer et al., 1999; Fang et al., 1999; Fung et al., 1998; Kishino et al., 1997; Lossie et al., 1998; Malzac et al., 1998; Matsuura et al., 1997; Minassian et al., 1998; Moncla et al., 1999a; Russo et al., 2000; van den Ouweland et al., 1999). Furthermore, maternal transmission of a targeted disruption of *Ube3a* resulted in a mouse that displayed several features of AS including motor dysfunction, inducible seizures and learning deficits (Jiang et al., 1998).

The ubiquitin pathway is a very important cellular process, controlling the degradation of almost every type of protein, including regulators of the cell cycle, transcription factors, tumor suppressors and DNA repair enzymes (Ciechanover et al., 2000). In addition, ubiquitination is involved in the etiology of several diseases. Angelman syndrome, caused by lack of the E3 ubiquitin protein ligase gene, was the first human disease attributed to mutations in the ubiquitination pathway. Other diseases now ascribed to defects in ubiquitination include Liddle's syndrome, a severe form of familial hypertension and several specific cancers. In addition, evidence suggests that the ubiquitination pathway is involved in the pathogenesis of Spinocerebellar Ataxia type-1, DiGeorge Syndrome and Cystic Fibrosis as well as late onset Alzheimer's, Parkinson's and Huntington diseases.

The process of protein ubiquitination is complex, specific and rapid. Multiple genes, which utilize different ubiquitin pathways, give the system its specificity and complexity (Ciechanover et al., 2000). The E3 enzymes, which are the most diverse, consist of at least six different families; each family processes proteins in slightly different ways. The gene responsible for Angelman syndrome, *UBE3A*, is involved in

the transfer of an activated ubiquitin molecule to the protein substrate. UBE3A is the founding member of the HECT-domain (homologous to E6AP carboxyl terminus) family of E3 ligases. These proteins act as intermediaries between the E2 ubiquitin conjugating enzymes and the targeted substrate. This suggests that accumulation of a specific protein in certain regions of the brain is the main cause of the AS phenotype. However, patients with AS do not demonstrate a phenotype consistent other protein storage disorders.

UBE3A is involved in the transfer of ubiquitin moieties to proteins targeted for degradation via the 26S proteosome. It was cloned in 1993 by its ability, in conjunction with the E6 oncoprotein of the cancer-associated human papillomaviruses (HPV), to deactivate the p53 tumor-suppressor protein (Huibregtse et al., 1993a). In HPV16 infected cells, the E6/UBE3A protein complex selectively bound p53 and targeted it for degradation via the ubiquitination pathway. Three functional domains of UBE3A have been identified: an E6 protein binding site, a p53 binding site and a HECT domain, which is necessary for ubiquitination (Huibregtse et al., 1993b).

Protein ubiquitination via the HECT family of protein ligases requires the combined efforts of multiple enzymes (Figure 1-6; Ciechanover et al., 2000). E1 activates the ubiquitin moiety, which then attaches to an E2 enzyme via a high-energy thioester bond. The E2 enzyme interacts with one HECT domain family member, transferring the ubiquitin molecule to the E3. The complex containing ubiquitin, as well as the E2 and E3 enzymes then follows one of two known paths. In the first pathway, the protein substrate binds the complex, and a single ubiquitin moiety is attached. In the alternative pathway, the complex requires the interaction of an ancillary protein, such as p53, for target recognition. With the help of the E4 enzyme,

the ubiquitin chains polymerize, targeting the proteins for degradation via the 26S proteosome (Koegl et al., 1999).

The following chapters describe my work on the molecular genetics and pathogenetic mechanisms of Angelman syndrome.

Figure 1-1. **Imprinted genes on 11p15.5.** This map illustrates the genes (top) that map to the Beckwith-Wiedemann syndrome region on human chromosome 11, with the imprinting status of the human genes depicted. The maternal (M) and paternal (P) alleles as well as expression profiles of biallelic (☐), maternal (▩) and paternal(■) genes are indicated. Arrows indicate location of transcription start site and direction of transcription. Figure not drawn to scale.

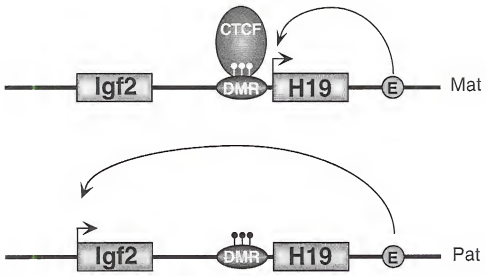


Figure 1-2. Genomic imprinting of *H19* and *Igf2* in the mouse. The *Igf2* and *H19* genes are shown. The differentially methylated region (DMR) of *H19* is located 5' to the transcriptional start site. On the maternal chromosome (Mat), the DMR is unmethylated (white lollipops), which facilitates binding of the CTCF protein. This interaction allows the enhancers (E) to interact with *H19*, but prohibits interactions between the enhancers and the *Igf2* promoter. On the paternal chromosome (Pat), the DMR is methylated. CTCF cannot bind, and the enhancers preferentially interact with the promoter of *Igf2*, inducing expression.

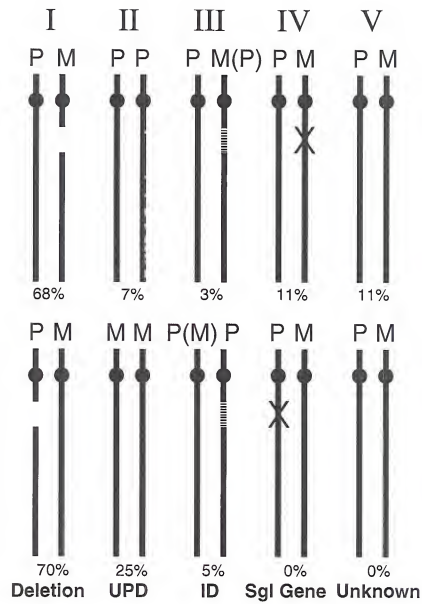


Figure 1-3. Molecular classes of AS and PWS in the UF repository. A total 93 AS families (104 patients) and 69 PWS families were analyzed. Familial cases were counted as one case per family. The maternal (M) and paternal (P) chromosomes are indicated. Percentages of patients in each molecular class are noted. **Class I)** 4Mb interstitial deletions of 15q11-q13; **Class II)** patients with uniparental disomy of chromosome 15; **Class III)** patients who have imprinting defects, which cause changes the epigenotype along 15q11-13; **Class IV)** patients with mutations in a single gene, *UBE3A* (X); **Class V)** patients with no detectable molecular defect.

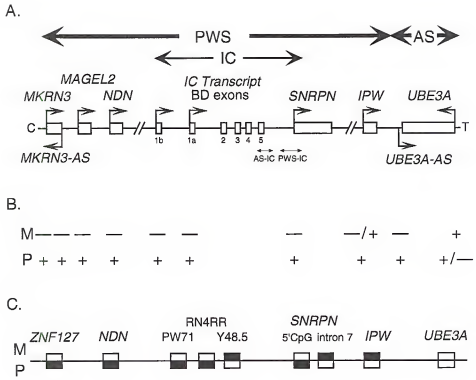


Figure 1-4. DNA methylation and expression imprints in 15q11-13. This figure depicts the DNA methylation and expression imprints of several imprinted genes on 15q11-13. **A)** Overview of the AS/PWS imprinted domain. The chromosomal regions of the Prader-Willi and Angelman syndromes are indicated by bold arrows (top). The extent of the imprinting center (IC) is also shown. The AS/PWS domain is depicted as a line, with the orientation of the centromere (C) and telomere (T) noted (not to scale). Relative positions of genes and transcripts are indicated above the line, while antisense transcripts are shown below. Arrows demonstrate transcriptional orientation. The SROs for the AS-IC and PWS-IC are shown below the chromosome. **B)** Expression imprints of selected 15q11-13 genes. Overall imprinted expression profiles of the maternal (M) and paternal (P) alleles are depicted. Active (+) and silenced (—) chromosomes are indicated. Tissue-specific imprinting is also indicated (+/—). **C)** DNA methylation imprints of select loci along the AS/PWS imprinted domain. Maternal imprints (M) are shown above the solid line, while paternal imprints (P) are revealed below. Gene and locus names are shown. Methylated (■) and unmethylated (□) alleles are indicated. This figure does not distinguish between partially and fully methylated loci.

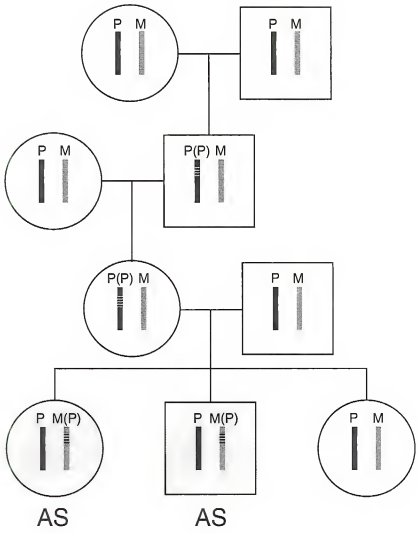


Figure 1-5. Inheritance of an imprinting defect. Silent transmission and inheritance of an imprinting defect in Angelman syndrome over four generations. Females (circles) and males (squares) are shown in each generation. Paternal (P) chromosomes are black, while maternal (M) chromosomes are grey. Imprinting mutations are depicted with appropriate epigenotypes in parenthesis. Affected siblings are denoted with a grey circle or box.

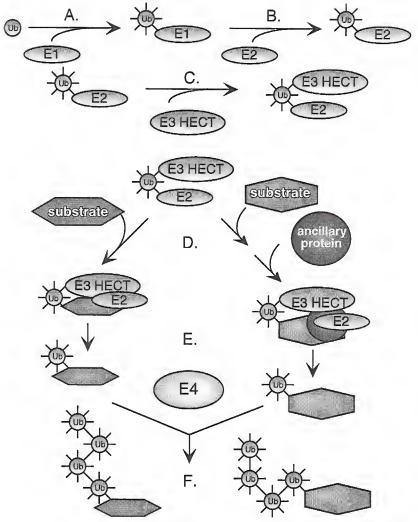


Figure 1-6. The ubiquitination pathway involving the HECT family of ubiquitin protein-ligases. **A)** Ubquitin is activated and bound by the E1 ubiquitin-activating enzyme. **B)** The E1 enzyme transfers the activated ubiquitin to an E2 ubiquitin-conjugating enzyme. Several E2 enzymes are known to interact with *UBE3A*. **C)** A member of the HECT family binds to its E2 conjugating enzyme. **D)** The active ubiquitin complex either recognizes a specific substrate or requires the help of an ancillary protein (e.g. the E6 protein from HPV) to bind its substrate. **E)** Once bound to its target protein, the ubiquitin-E2-E3 complex transfers the activated ubiquitin moiety to its substrate. **F)** The E4 protein then aids in polymerization of ubiquitin molecules on the substrate. The protein then is degraded via the 26S proteosome.

TRANSMISSION OF ANGELMAN SYNDROME BY AN AFFECTED MOTHER

Introduction

The Angelman syndrome (AS) is a severe developmental and neurobehavioral disorder characterized by four cardinal features: 1) severe developmental delay; 2) profound speech impairment; 3) a movement and balance disorder and 4) an AS-specific behavior which includes frequent and inappropriate laughter, a happy affect and an easily excitable personality (Williams et al., 1995a; Williams et al., 2000). Most AS cases arise *de novo*, the consequences of large deletions from the maternally derived 15q11-13 (Williams et al., 2000). AS has also been diagnosed in patients with paternal uniparental disomy, imprinting defects on maternal 15q11-13 and maternally inherited mutations in the 3A ubiquitin protein ligase gene, *UBE3A* (Glenn et al., 1997). The Prader-Willi syndrome (PWS) is a separate, distinct neurobehavioral syndrome also associated with 15q11-13 deletions. However, the deficiency in PWS is always paternal in origin (Glenn et al., 1997). All of the cardinal features of AS can be caused by mutations in the *UBE3A* gene (Kishino et al., 1997; Matsuura et al., 1997), which shows imprinted expression in specific regions of the mammalian brain (Albrecht et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997).

Individuals with AS achieve puberty normally, with both males and females having normal secondary sexual characteristics, including normal menses in females (Williams et al., 1995b). However, there have been no documented cases of reproduction in either males or females with AS. In this report, we present the first

documented case of reproduction in an individual with AS that resulted in the maternal transmission of a 15q11-13 deletion to a fetus, which was electively terminated at 16 weeks gestation (Lossie and Driscoll, 1999).

Methods

Subjects

The proband is now a 15 year old female with AS who has been followed intermittently in the UF Genetics clinic since 22 months of age. Recently, the family recontacted the Genetics service due to the proband's unexpected pregnancy. High-resolution chromosomal analysis in the past demonstrated a 15q11-13 deletion in the proband, which DNA polymorphism and methylation studies subsequently confirmed (Williams et al., 1989).

This family was initially reported in 1989 when the concern was raised that the proband's mother, who was of normal intelligence, could be mosaic for or also have a submicroscopic deletion of 15q11-13 because she displayed brachycephaly, hearing loss, an enlarged foramen magnum and mild ataxia. However, extensive molecular analyses of peripheral blood and skin fibroblasts failed to reveal any abnormalities in 15q11-13 in the mother of the proband.

The proband has classic AS features with severe mental retardation, an AS-specific behavior, a complete lack of speech, and a movement disorder characterized by ataxia. An abnormal EEG and seizures were also noted at 22 months. She began walking at 3 years, and her severe truncal ataxia has improved with age. The proband also has microcephaly with a head circumference of less than -2 standard deviations, relative prognathism, a protruding tongue, excessive drooling and an inappropriately happy affect with excessive laughter. Menarche began at 11.5 years. A head CT and MRI were only remarkable for an enlarged foramen magnum.

The family decided to terminate the pregnancy at 15-16 weeks gestation. The termination was by suction dilatation and curettage. Identifiable fetal tissue was dissected, immediately frozen in liquid nitrogen and stored at -70°C. Due to the suction procedure, the only intact brain samples obtained were the eyes. Tissue was procured with the approval of the family and the University of Florida Institutional Review Board.

Microsatellite Analysis

DNA was extracted from peripheral blood leukocytes (PBL) and whole tissue according to standard techniques (Driscoll and Migeon, 1990). PCR amplification of the *D15S817*, *D15S128*, *ACTIN* (*ACTC*), *D15S175* and *D15S87* loci (Research Genetics) was as previously described (Colman et al., 1996).

DNA Methylation Analyses

Southern Blot. DNA methylation analyses were performed within 15q11-13 at *SNRPN* (Glenn et al., 1996) and *NECDIN* (*NDN*), and in 11p15 (KvDMR1; Smilinich et al., 1999) according to published protocols. DNA methylation analysis of *NDN* was at a novel *Bss* HII site (AC Lossie, RD Nicholls, DJ Driscoll, unpublished data), which is located within the same *Hind* III fragment as the published imprinted *Eag* I site (Jay et al., 1997). Specific conditions for each locus are shown in Table 2-1.

Table 2-1. Conditions for Southern blots

Gene	µg DNA	RE	RE	% Gel	V•hrs	Wash °C
SNRPN	5	*Xba* I	*Not* I	0.8	550	65
NECDIN	10	*Hind* III	*Bss* HII	1	1000	70
KvLQT1	5	*Eco* RI	*Not* I	1	850	65

Specific restriction enzymes (RE) and volt•hours (V•hrs) for each locus are noted

Methylation Specific PCR (MSP). MSP relies on the selective ability of sodium bisulfite to convert unmethylated cytosine to uracil, while leaving methylated cytosine untouched. During PCR amplification, uracil is converted to thymidine, providing a method to distinguish between the methylated and unmethylated alleles. MSP at 5' *SNRPN* was done according to the manufacturers recommendations (Oncor) using primers developed by Kubota and colleagues (Kubota et al., 1997).

RT-PCR

mRNA was isolated from fetal eye and lymphoblast cell lines using the Polytract System 1000 mRNA extraction kit (Promega). cDNAs were reverse-transcribed from 20-200 ng of mRNA using the Superscript™ Amplification kit (Gibco). RT-PCR analysis of *UBE3A* was accomplished using previously published primers that amplify an 1150 bp fragment from exons 3 to 9 (formerly U1 to 3) of *UBE3A* (Matsuura et al., 1997). A 738 bp fragment of *SNRPN* was amplified using primers specific for exon 2 (5'-TTGGCACACCAGCTGGTACT-3') through exon 5 (5'-CACCTGAGACGAACTACAG-3') in a standard reaction with a 57°C annealing temperature. Amplification products were size fractionated by gel electrophoresis, Southern blotted and visualized by autoradiography.

Results

Genetic Analysis

The proband, fetus and parents of the proband were genotyped by microsatellite analysis of loci (see map Figure 2-1A) within (*D15S817* and *D15S128*; Christian et al., 1998) and distal to 15q11-13 (*ACTIN*, *D15S175* and *D15S87*). Within 15q11-13, the proband and fetus lacked a maternal genetic contribution (Figure 2-1B). At *D15S128*, the proband and fetus only amplified the paternally inherited allele.

Although the proband was non-informative at *D15S817*, the fetus showed exclusive amplification of the paternally derived allele. However, the proband and fetus were heterozygous at several loci distal to 15q13, including *D15S175*, *ACTIN* (Figure 2-1C) and *D15S87* (data not shown). Therefore, both the proband and fetus have maternally inherited deletions which are restricted to 15q11-13.

DNA Methylation Analyses

DNA methylation analysis is a powerful diagnostic tool that takes advantage of the differential methylation found in imprinted regions of the genome. The AS/PWS imprinted domain contains several loci that display parent-of-origin methylation imprints (Glenn et al., 1997). *SNRPN* and *NDN* are two of several paternally expressed genes within 15q11-13 which are preferentially methylated on the repressed maternal allele (Glenn et al., 1996; Jay et al., 1997; Sutcliffe et al., 1994).

Two methods can assess DNA methylation at 5' *SNRPN*: Southern analysis and MSP (Glenn et al., 1996; Kubota et al., 1997; Kubota et al., 1996b; Sutcliffe et al., 1994). Southern analysis reveals a 4.3 kb maternal and a 0.9 kb paternal band in the parents and normal controls. However, the proband and fetus only have the 0.9 kb paternal band (Figure 2-2), confirming the lack of a maternal 5' *SNRPN* allele in these samples. In accordance with Southern analysis, MSP analysis of parental PBL and normal fetal eye DNA shows both the maternal and paternal PCR amplification products. However, PBL DNA from the proband and eye DNA from the fetus exclusively amplified the paternal chromosome 15 (Figure 2-3).

Previous methylation studies of the CpG island associated with *NDN* revealed a parent-specific DNA methylation imprint from DNA digested with *Hind* III and *Eag* I (Jay et al., 1997). A *Bss* HII methylation imprint is also present in this 2.6 kb *Hind* III fragment (AC Lossie, RD Nicholls, DJ Driscoll, unpublished data). *NDN* hybridizes to

three fragments in control samples: a 2.6 kb maternal-specific, a 1.7 kb constant and a 1.3 kb paternal-specific fragment (Figure 2-4). Normal PBL and fetal eye DNA reveal both the maternal and paternal diagnostic bands. However, PBL DNA from individuals with PWS reveals the predominance of the methylated 2.6 kb fragment, while *NDN* hybridized to the paternal-specific unmethylated 1.3 kb fragment in patients with AS. DNA from the parents of the AS proband demonstrates normal biparental methylation, while AS methylation imprints are apparent in PBL DNA from the proband with AS and eye tissue from the fetus with AS.

A differentially methylated region near an antisense transcript of the *KvLQT1* gene in the Beckwith-Wiedemann syndrome region on chromosome 11 was also examined (Smilinich et al.,). Normal individuals have 4.3 and 2.7 kb diagnostic bands, which correspond with the methylated and unmethylated alleles, respectively. All of our samples showed normal DNA methylation imprints at this non-chromosome 15 differentially methylated site (data not shown).

Gene Expression

RT-PCR analyses of AS fetal eye, normal fetal eye and PWS lymphoblast cells revealed a 1.4 kb transcript from exons 4 to 9 of *UBE3A* in all tissues (Figure 5). However, as expected, *SNRPN* was only expressed in the AS and normal tissues; the PWS sample failed to amplify *SNRPN* (Glenn et al., 1996).

Discussion

The presence of AS-specific DNA methylation imprints and absence of a maternal contribution of proximal 15q demonstrates that the patient with AS passed her 15q11-13 deletion to her fetus. These findings conclusively demonstrate that females with AS are capable of reproduction and show that the previously observed

lack of reproduction is most likely due to social/cognitive behavior versus physiological reasons. Therefore, appropriate appraisal of the reproductive risks should be part of the genetic counseling given to AS families.

Studies by Gabriel and colleagues (Gabriel et al., 1999) of a mouse deletion model for the AS/PWS imprinted region support our findings. Their model, a transgene insertion/deletion ($Tg^{PWS/AS(del)}$) of the orthologous region on mouse chromosome 7C, correlates well with our findings in this patient with AS, as a large murine chromosomal deletion can also be passed through the maternal germ line. Mice that inherit the maternal deletion have many of the characteristics found in individuals with Angelman syndrome.

The ability of an AS mouse to pass on either a normal or deleted chromosome 7C allows a closer examination of the role that replication timing and homologous association play in the establishment and transmission of the chromosome 15q11-13 imprint (Kitsberg et al., 1993; LaSalle and Lalande, 1996). In the mouse model for AS, maternal transmission of a large deletion encompassing the imprinted domain failed to interrupt proper imprint establishment of the normal AS/PWS chromosomal region (Gabriel et al., 1999). These findings demonstrate that during murine gametogenesis the imprinting on both homologues must be established and transmitted in *cis*, and that neither replication timing nor homologous association are required for establishment or transmission of the imprint. In addition, these data strongly suggest that inheritance of a normal chromosome 15 from a mother with AS would result in normal development in her offspring.

Evidence for imprinted expression of the *UBE3A* gene is limited. Murine studies demonstrate that imprinted expression is limited to the CA2 and CA3 regions of the hippocampus, the mitral cell layer of the olfactory bulb and Purkinje cells of the

cerebellum (Albrecht et al., 1997). In humans, evidence is less precise. Imprinted expression of *UBE3A* was observed in adult frontal cortex and fetal brain (Rougeulle et al., 1997; Vu and Hoffman, 1997). Since the eyes originate from the lateral aspects of the forebrain during fetal development, it was possible that fetal eye would also show imprinted expression. However, no difference in the expression of *UBE3A* was observed between normal and AS fetal eye samples.

Imprinted genes in other chromosomal regions also show DNA methylation imprints (Razin and Cedar, 1994). To determine if interruption of the imprinting process in the AS/PWS region can alter DNA methylation imprints at loci on 11p15, we examined KvDMR1, a differentially methylated site associated with an antisense transcript of *KvLQT1* (Smilinich et al., 1999). All samples showed normal DNA methylation, indicating that the establishment and transmission of the 11p15 DNA methylation imprint occurs independently from the AS/PWS region.

In conclusion, our findings establish that the Angelman syndrome can be transmitted through a female with AS, imply that the Prader-Willi syndrome could also be transmitted through a male with AS and illustrate the need for informed reproductive counseling for parents of AS patients.

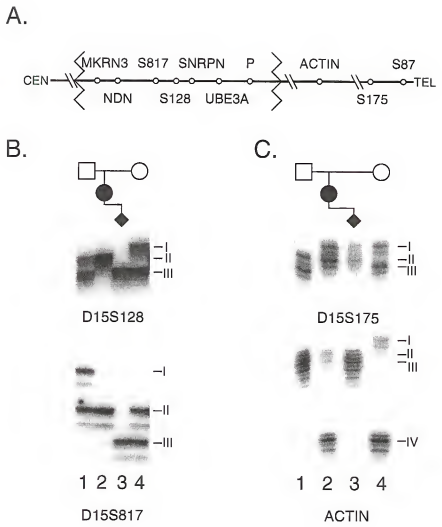


Figure 2-1. Microsatellite Analysis of Chromosome 15. A) deletion breakpoints flanking 15q11-13, individual loci, centromere (CEN) and telomere (TEL) are noted. B) Microsatellite analysis of loci within 15q11-13 and C) Microsatellite analysis of loci distal to 15q11-13. The pedigree shows the father of the proband (lane 1), AS proband (lane 2), proband's fetus (lane 3) and mother of the proband (lane 4). Note the lack of a maternal contribution within 15q11-13 for the proband and fetus at *D15S128*, and for the fetus at *D15S817*. However, distal to 15q11-13, there is a biparental contribution at *ACTIN* and *D15S175*. The father of the AS fetus is unknown.

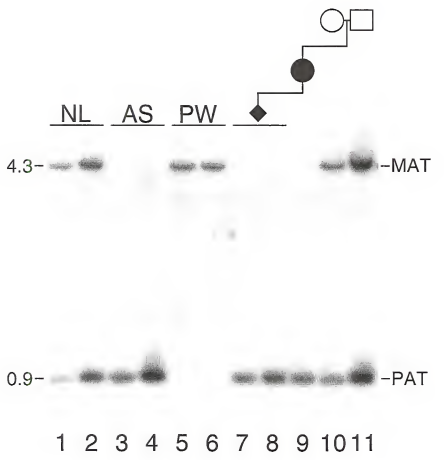


Figure 2-2. DNA methylation analysis of 5' *SNRPN*. Digestion of DNA with *Xba* I and *Not* I reveals diagnostic bands of 4.3 kb (methylated, maternal) and 0.9 kb (unmethylated, paternal). PBL DNA from normal individuals reveals both the 4.3 kb and 0.9 kb normal methylation profile (lanes 1, 2, 10, 11). Patients with AS only have the 0.9 kb unmethylated fragments (lanes 3 and 4), while individuals with PWS have the 4.3 kb methylated allele (lanes 5 and 6). Eye DNA from the fetus (lanes 7 and 8) and PBL DNA from the AS proband (lane 9) reveal AS-specific DNA methylation imprints, while PBL DNA from the parents shows maternal- and paternal-specific bands (lanes 10 and 11).

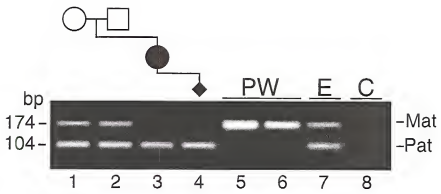


Figure 2-3. MSP analysis of 5' *SNRPN*. PBL (lanes 1, 2, 3, 5, and 6) and fetal eye DNAs (lanes 4 and 7) are shown. The pedigree shows the positions of each family member. Normal individuals (lanes 1, 2 and 7) amplified the 174 bp methylated and 104 bp unmethylated products. The proband and fetus exclusively amplified the unmethylated allele, while two patients with PWS (lanes 5 and 6) amplified only the methylated allele. PW (Prader-Willi), normal fetal eye (E) and water control (C) are indicated.

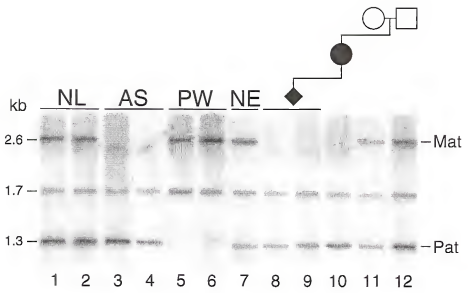


Figure 2-4. DNA methylation analysis of *NDN*. Digestion of DNA with *Bss* HII and *Hind* III reveals diagnostic bands of 2.6 kb (methylated, maternal) and 1.3 kb (unmethylated, paternal), and a constant band of 1.7 kb. PBL DNA from normal individuals (lanes 1, 2, 11, 12) and normal fetal eye (lane 7) shows the normal methylation profile. Patients with AS (lanes 3 and 4) have the 1.3 kb paternal diagnostic band, while individuals with PWS (lanes 5 and 6) predominantly have the 2.6 kb methylated, maternal fragment. Eye DNA from the fetus and PBL DNA from the proband reveal AS-specific DNA methylation imprints (lanes 8, 9 and 10).

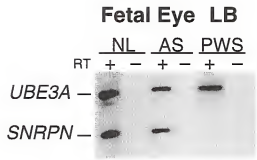


Figure 2-5. Expression analysis of *UBE3A* and *SNRPN*. A Southern blot of RT-PCR products from *UBE3A* exons 3 to 9 (1150 bp) and *SNRPN* exons 2 to 5 (738 bp) is shown. The membrane was hybridized with the same RT-PCR products. Reactions with (+) and without (-) RT are noted. *UBE3A* and *SNRPN* transcripts are present in normal (lanes 1 and 2) and AS fetal eye (lanes 3 and 4). However, only *UBE3A* is expressed in lymphoblast cells from a patient with PWS (lanes 5 and 6). Extra bands are due to alternative splicing of *UBE3A*.

MOLECULAR ANALYSES AND GENOTYPE-PHENOTYPE-CORRELATIONS AMONG THE FIVE CLASSES OF ANGELMAN SYNDROME

Introduction

The Angelman (AS) and Prader-Willi (PWS) syndromes, two distinct neurobehavioral disorders, are the best-studied examples of genomic imprinting in humans. In these imprinted disorders, the parental origin of the chromosome 15 abnormality determines the phenotype. PWS occurs from the lack of critical paternally expressed 15q11-13 sequences, while multiple mechanisms that disrupt the maternally inherited E3 ubiquitin protein-ligase gene (*UBE3A*) cause AS (Glenn et al., 1997; Williams et al., 2000).

AS is a severe developmental and neurobehavioral disorder that occurs with a frequency of ~1/15,000 (Clayton-Smith et al., 1993). First documented by Harry Angelman (1965), AS is now diagnosed by four cardinal features: severe developmental delay and mental retardation; profound speech impairment; a movement and balance disorder and an AS-specific behavior which includes frequent, inappropriate laughter, a happy affect and an easily excitable personality. Other common features include seizures, microcephaly, abnormal EEG patterns, sleep disturbances, hypopigmentation and strabismus (Williams et al., 1995a; Williams et al., 2000).

Five major molecular mechanisms are known to cause AS: I) 4 Mb maternally-derived deletions of 15q11-13; II) paternal uniparental disomies (UPD); III) imprinting defects (ID); IV) patients with intragenic mutations in *UBE3A*; and V) patients with no

known defects on chromosome 15. Although patients in Classes I-III can be easily diagnosed by DNA methylation analyses along the 15q11-13 imprinted domain (Glenn et al., 1997), individuals from Classes IV and V demonstrate normal DNA methylation at the diagnostic loci. DNA methylation analysis takes advantage of the differential methylation found in imprinted regions of the genome and easily resolves the maternal and paternal alleles. *SNRPN*, the most reliable locus for AS diagnosis, is one of several paternal-only expressed genes in 15q11-13 whose promoter region is preferentially methylated on the repressed maternal allele (Glenn et al., 1996; Kubota et al., 1996b; Sutcliffe et al., 1994).

The discovery of maternally inherited mutations in Class IV patients first established a role for *UBE3A* in the etiology of AS (Kishino et al., 1997; Matsuura et al., 1997). Subsequent mutation analysis of patients with normal DNA methylation identified an overall *UBE3A* mutation rate of 14-38% in sporadic AS patients (Baumer et al., 1999; Fang et al., 1999; Malzac et al., 1998; Moncla et al., 1999a; Russo et al., 2000). Although cell lines from AS patients failed to show imprinted expression of *UBE3A* (Nakao et al., 1994), demonstration of brain-specific imprinting by RT-PCR in the human confirmed the role of *UBE3A* in the etiology of AS (Rougeulle et al., 1997).

The phenotype associated with Class I patients is well documented (Saitoh et al., 1994; Smith et al., 1996). These individuals have a classical AS phenotype with all patients having the four cardinal features of AS as well as a very high incidence of severe, early-onset seizures, microcephaly and hypopigmentation. While patients from Classes II, III and IV displayed the four cardinal features of AS, these individuals often exhibited atypical features that ameliorated the AS phenotype. All studies showed that patients with UPD and imprinting defects (ID) were less likely to present with hypopigmentation or microcephaly (Burger et al., 1996; Fridman et al., 2000b;

Gillessen-Kaesbach et al., 1999; Saitoh et al., 1999; Smith et al., 1997). In addition, some studies revealed that patients from Classes II and III had fewer seizures, early onset obesity and better motor skills (Fridman et al., 2000b; Gillessen-Kaesbach et al., 1999; Smith et al., 1997). Moncla and colleagues (1999b) reported that *UBE3A* mutation patients had a more severe phenotype than the UPD and ID patients. Like the deletion group, Class IV patients were more likely to present with hypopigmentation and seizures.

Individually, these studies have given us clues to the clinical variances that occur among the first four classes of AS patients. However, a single comprehensive investigation of all molecular classes of Angelman syndrome would provide researchers, clinicians and families a valuable resource to aid in the prediction of a phenotypic outcome give a particular genotype or molecular class. In this study, we report on the molecular and clinical analyses of 104 classical AS patients from the University of Florida AS repository. We present the first comprehensive analysis of genotype-phenotype correlations among all five molecular classes of AS. In our study, we report the highest *UBE3A* mutation frequency to date and demonstrate striking differences among the five classes in growth parameters; achievement of developmental milestones; and severity, frequency and age at onset of seizures.

Materials and Methods

Patient Repository

Over the last 11 years, we have accumulated a large AS repository totaling 146 individuals from 134 Angelman families. Each patient was clinically examined by geneticists at the University of Florida, evaluated for 22 individual criteria and given an AS rating of one through five based on clinical impression before any laboratory tests were performed. We restricted this study to the 104/146 classical AS patients

(93 families) who demonstrated a classical AS phenotype. The remaining 42 "AS-like" patients exhibit some but not all of the four cardinal features of AS and were excluded from further investigation. Blood and tissue were procured with the approval of the families and the University of Florida Institutional Review Board.

Once a clinical diagnosis of AS was determined, DNA from each patient was analyzed by molecular and cytogenetic tests in order to determine the class in which each AS patient belonged. DNA methylation analyses at the *MKRN3*, PW71 and 5' *SNRPN* loci were used to separate patients in Classes I-III (abnormal DNA methylation) from individuals in Classes IV and V, who had normal methylation (Dittrich et al., 1993; Driscoll et al., 1992; Glenn et al., 1996; Kubota et al., 1996b; Sutcliffe et al., 1994). DNA dosing, high-resolution chromosome and fluorescence *in situ* hybridization (FISH) studies detected patients with large interstitial deletions (Class I), while microsatellite and RFLP analyses separated patients in Class II from individuals in Class III (Christian et al., 1998; Miturangura et al., 1993).

Patients with normal DNA methylation were then tested for *UBE3A* mutations by a combination of Southern blot, BESS T-Scan and direct sequence analyses. Patients with intragenic *UBE3A* mutations were placed into Class IV, while those with no detectable chromosome 15 abnormalities were put into Class V.

Southern Analysis

Southern blots were done according to standard conditions (Lossie and Driscoll, 1999). To detect possible genomic rearrangements, cDNA probes were made by RT-PCR from the 5' *UBE3A* cDNA primers (forward primer 5'-ACCAGCTCCTCAGAAGTTT GGCGAAA-3' and reverse primer 5'-TCTCCATCATTCTCCGAATCTGGTC-3') and 3' *UBE3A* cDNA primers (forward primer 5'-TTGCAAAGCGATGAGCAAGCTACC-3' and reverse primer 5'-TGGGACACTATCACCACCCAA-3'). A 2.7 kb *Eco* RI to *Not* I fragment from

cosmid 24 (Sutcliffe et al., 1994), which is located at 5' *UBE3A*, was used for DNA methylation analyses and to detect genomic rearrangements.

BESS T-Scan

We analyzed genomic DNA from patients following the procedures outlined in the BESS T-Scan mutation detection kit (Epicentre, Madison, WI) using primers (Table 3-1) corresponding to exons 6, 10, 11, 12, and 16. Cleaved products were size fractionated on 6% acrylamide, 6M urea and 30% (v/v) formamide sequencing gels according to standard protocols. Putative mutations were verified by sequence analysis.

Sequence Analysis

Abnormal PCR products from BESS T-scan analyses were cloned into a TA vector (Clontech) and sequenced using universal primers and regular dye terminators (ABI, Perkin Elmer). We designed coding region and intronic primers that would amplify individual exons of *UBE3A* (Table 3-2). PCR products were purified by the High Pure PCR Purification Kit (Roche) and sequenced directly using a 1/4 reaction of the Big Dye terminators (ABI, Perkin Elmer). Completed sequencing reactions were purified by either repeated ethanol precipitation or by sepharose columns (Edge Scientific), dried under vacuum, resuspended and fractionated on either an ABI 377 or ABI 373A automated sequencer. Sequence analysis was performed with the Sequencher program (Gene Codes Corporation).

Primers

Primers for BESS-T (Table 3-1) and direct sequence analysis (Table 3-2) were designed to amplify the entire *UBE3A* coding region (exons 6-16).

Table 3-1. BESS-T Primers

Exon	Primer	Direction	Location	Size
6	TGCCAGCAGGTTTATTTTT	Forward	Exon	187
	ACCAGCCTTGTGGGTAAG	Reverse	Exon	
7	GGAGAACCTCAGTCTGACGAC	Forward	Exon	38
	ATTCGGCTAGCTTCAATGTC	Reverse	Exon	
8	GCAGCTGCAAAGCATCTAAT	Forward	Exon	284
	TCAATTCTAGCGCCTTTCTT	Reverse	Exon	
10	GCAATCATCTTCTTTTCATGTT	Forward	Intron	194
	CGACACCATAATCACATTAC	Reverse	Intron	
11	TGTTCACATACGATGAATCTACA	Forward	Exon	192
	CTCCCAAGTCACGAAAAGTT	Reverse	Exon	
12	TCAGAGTTTAAAAGATTTATTGGA	Forward	Exon	155
	TCCTGTTTTCATTTGTAATTGG	Reverse	Exon	
13	GAATTTGTCAATCTTTATTCTGACT	Forward	Exon	155
	CGGCTTCCACATATAAGCA	Reverse	Exon	
14	AGATTTCCAAGCACTAGAAGAAA	Forward	Exon	67
	AATCAGAACAGAGTCCCTGG	Reverse	Exon	
15	GTTCTGGGAAATCGTTCATT	Forward	Exon	137
	CTGTGTCTGGGCCATTTT	Reverse	Exon	
16	ACCATGACTTACAGTTTTCCT	Forward	Intron	189
	TGGGACACTATCACCACCAA	Reverse	Intron	

Table 3-2. Sequencing Primers

Exon	Primer	Direction	Location	Size
7	TATGGCCACCTGATCTGAC	Forward	Intron	345
	TTCCTATCTCCCATTTACTGC	Reverse	Intron	
8	GCTTGACTAACTTTTGCCTTG	Forward	Intron	443
	ATCTCCCACATGGTTTTCAG	Reverse	Intron	
	GCAGCTGCAAAGCATCTAAT	Forward	Exon	
	TCAATTCTAGCGCCTTTCTT	Reverse	Exon	
9	AACAGCTATGACCATGCAACAGAGTAAACATACATATT	Forward	Intron	1401
	GTAAAACGACGGCCAGTCACTGAACTGTATCAT	Reverse	Intron	
	ATTAGGCCCTGATGATGTGT	Forward	Exon	
	CCAGATATTCAGGACTGTGGA	Reverse	Exon	
10	TTGTTTCCTAATCCTACTCCTTG	Forward	Intron	352
	ACCAAATCCTTCTTTTGCTG	Reverse	Intron	
11	GGACATTGTTTTCTCAAGTGC	Forward	Intron	559
	TAAAAATGTCCCCCTTTGAG	Reverse	Intron	
12	TGTTGTATTTTGTAGTTCTATGG	Forward	Intron	257
	TTAATGAAGAGACAAAATGTGAC	Reverse	Intron	
13/14	GAAGTTCTTGTGATTAATGT	Forward	Intron	404
	CCCTTTGGTGAATCAAATCTTCC	Reverse	Intron	
	GAATTTGTCAATCTTTATTCTGACT	Forward	Exon	
	AATCAGAACAGAGTCCCTGG	Reverse	Exon	
15	TTCAGGATAAATTTGCTTGG	Forward	Intron	416
	AAAATCACGAATGTGCTCAG	Reverse	Intron	

Statistical Analyses

Descriptive statistics were calculated as a percentage or by the mean +/- standard deviation. All outcome variables were modeled as a function of age at evaluation, birth weight, molecular class (I, II, III, IV and V), gestational age, maternal

age, paternal age, race (Caucasian, Hispanic and Other), and sex. Outcome variables consisted of continuous, binary and ordinal measures, and were modeled using analysis of covariance (ANCOVA), logistic regression and ordinal logistic regression, respectively. For binary and ordinal outcome variables with limited sample size, we employed the exact chi-square test. Odds ratios were used to summarize the binary and ordinal outcomes. All tests were two-sided and considered significant at alpha=0.05. All statistical analyses were performed using SAS 6.12 (SAS Institute, Cary, NC).

Results

Molecular Classes of Angelman Syndrome

Each patient was assigned a molecular class based upon results from high-resolution chromosome, FISH, *UBE3A* mutation as well as DNA dosing, methylation and polymorphism analyses along 15q11-13 (Table 3-3). The vast majority (63) of our

Table 3-3. Molecular Classes of Angelman Syndrome

Class	Molecular Defect	Patients	%
I	15q11-13 Deletion	63	68
II	Uniparental Disomy (UPD)	7	7
III	Imprinting Defect (ID)	3	3
IV	*UBE3A* Mutation	10	11
V	Unknown	10	11

classical families were deleted for the entire 4 Mb AS/PWS imprinted domain (Class I). In seven cases AS occurred via paternal UPD of chromosome 15 (Class II), and three families had imprinting defects (Class III). Intragenic mutations in *UBE3A* occurred in ten of our classical patients (Class IV), while the remaining 10 had no detectable chromosome 15 abnormality (Class V).

Mutation Analysis

Initially, a molecular confirmation of AS could not be made in 20/93 families using cytogenetic, DNA methylation or DNA polymorphism tests. Furthermore, Southern blot analysis of the *UBE3A* cDNA determined that genomic rearrangements did not occur in any of these patients (data not shown). Sequence analysis of the *UBE3A* coding region in these 20 families revealed intragenic mutations in 10 families (15 patients). We detected six deletions that resulted in frameshifts, three nonsense mutations and one missense mutation (Figure 3-1). In patients with normal DNA methylation, we found a 50% mutation detection rate with 3/4 familial (75%) and 7/16 sporadic cases (44%) harboring mutations within the *UBE3A* gene.

One patient was heterozygous for a previously unidentified expressed polymorphism (A1144G transition) in exon 9. Another common polymorphism occurred in intron 6 at the site of a poly T tract. Several patients and normal individuals were heterozygous, having runs of either nine (p) or ten (q) thymidines at in intron 6. We examined 134 normal chromosomes and found that p=0.83 and q=0.17. In addition, one AS-like patient had a paternally inherited 14 bp deletion of the 3' UTR, which appeared to be similar, but not identical, to that identified by Fung and colleagues (1998). No disease-associated mutation was found in this individual. In addition, BESS T-Scan detected one individual who was homozygous for a previously identified expressed polymorphism in exon 6 (Vu and Hoffman, 1997).

DNA Methylation of 5' *UBE3A*

Although we identified disease-causing mutations in 50% of our patients who had normal DNA methylation at 5' SNRPN, the cause of AS in the remainder of our classical Angelman syndrome patients was unknown. We postulated that disruption of

the normal *UBE3A* DNA methylation pattern in Class V patients could be a potential cause of AS in these patients.

We isolated a 2.7 kb *Not* I-*Eco* RI fragment from the CpG island at 5' *UBE3A* (Figure 3-2A) and tested for parent-of-origin DNA methylation imprints in DNA from PBL, AS brain, normal brain and germ cells from both sexes. As expected, the 2.7 kb genomic probe detected a 5.1 kb fragment when the DNA was digested with *Eco* RI alone. However, when DNA was digested with both *Eco* RI and *Not* I, the probe only hybridized to a 2.7 kb fragment, indicating that the CpG island at 5' *UBE3A* was unmethylated on both alleles in all tissues examined (Figure 3-2B). We next examined PBL DNA from Class V patients. The 2.7 kb probe only detected the unmethylated fragment, indicating that the CpG island was also unmethylated in these individuals (Figure 3-2C).

Summary of all Published *UBE3A* Mutations

To date, *UBE3A* mutations have been identified in 51 AS families (Baumer et al., 1999; Fang et al., 1999; Fung et al., 1998; Kishino et al., 1997; Malzac et al., 1998; Matsuura et al., 1997; Russo et al., 2000; van den Ouweland et al., 1999). Forty-five are unique mutations; no true hot spots have been identified (Figure 3-3). Most mutations are novel, with no more than three unrelated individuals sharing a common error. Although defects have been found in virtually all protein-coding exons, most cluster in exons 9 and 16 (Figure 3-3). Insertions, deletions and nonsense mutations are the predominant defects.

Genotype-Phenotype Correlations

We analyzed phenotypic data on 61 patients from the University of Florida AS repository. We included all of our patients from Classes II-V and randomly chose 20

Class I patients for phenotype comparisons. We also included 77 additional patients (Classes II, III and IV) in our statistical models (Bottani et al., 1994; Dan et al., 2000; Dupont et al., 1999; Freeman et al., 1993; Fridman et al., 2000a; Fridman et al., 2000b; Fridman et al., 1998; Fung et al., 1998; Gillessen-Kaesbach et al., 1995; Gillessen-Kaesbach et al., 1999; Greger et al., 1997; Gyftodimou et al., 1999; Laan et al., 1999; Malcolm et al., 1991; Minassian et al., 1998; Moncla et al., 1999a; Moncla et al., 1999b; Ohta et al., 1999; Prasad and Wagstaff, 1997; Reis et al., 1994; Robinson et al., 1993; Russo et al., 2000; Saitoh et al., 1997; Saitoh et al., 1999; Smeets et al., 1992; Smith et al., 1994; Smith et al., 1997; Smith et al., 1998; Tonk et al., 1996). We compared the results of statistical models established between our patients and the combined data set (Tables 3-4, 3-5 and 3-6). Deletion patients (Class I) had the most classical phenotype. However, patients from Classes II-V exhibited some atypical features. Differences were most striking in growth parameters, development of motor skills, seizures and pigmentation.

Statistical analysis of body mass index (BMI) revealed that patients from Classes II, III and IV were bigger and heavier than the deletions and Class V patients (Figure 3-4A; Table 3-4), for both UF patients (P=0.0042) and the combined data set (p=0.0167). Statistical models also demonstrated that less than 15% of individuals from Classes II and III had microcephaly, whereas more than 55% of patients from the other three classes had a head circumference less than the 3rd percentile (Figure 3-5B; Table 3-4; p=.0415).

The mean age to walk for Class I was 4.6 +/- 2.1 (p=.0001), while the mean age to walk for patients from Classes II-V varied from 2.4 +/- 0.9 (Class III) to 2.8 +/- 0.9 (Class IV; Figure 3-4C; Table 3-5). Onset of seizures also depended upon molecular class (Figure 3-4D; Table 3-5). Seizures started very early in Class I and V patients (1.9

+/- 1.1 and 1.4 +/- 0.9, respectively) compared to Classes II, III and IV (4.9 +/- 3.4, 5.5 +/- 5.0 and 2.7 +/- 1.4, respectively; p=.0028).

Table 3-4. Clinical and growth data

	Class I	Class II		Class III		Class IV		Class V
	UF	UF	All	UF	All	UF	All	UF
Speech	(21)	(7)	(27)	(7)	(20)	(15)	(46)	(11)
0 words	71	*	44	43	40	60	33	82
< 3 words	29	43	15	14	15	27	26	9
3-10 words	*	43	33	43	30	13	41	9
> 10 words	*	14	8	*	15	*	*	*
Seizure Onset	(20)	(3)	(14)	(2)	(9)	(12)	(24)	(5)
< 1 year	10	*	7	*	33	17	13	40
1-2 yrs	40	33	14	*	23	8	4	20
2-3 yrs	25	*	14	50	11	25	29	40
3-4 yrs	25	*	*	*	*	50	29	*
> 4 yrs	*	67	65	50	33	*	25	*
Seizure Severity	(21)	(7)	(26)	(7)	(18)	(15)	(32)	(10)
Severe	29	*	4	14	6	33	19	10
Moderate	62	14	8	*	11	20	22	30
Mild	9	29	23	14	22	33	44	20
None	*	57	65	72	61	14	15	40
Length	(21)	(7)	(28)	(7)	(17)	(15)	(30)	(9)
< 5 %	42	*	7	30	12	7	10	*
5-20%	5	*	11	14	12	20	10	56
21-40%	14	43	18	14	18	46	27	22
41-60%	19	14	14	14	23	13	33	*
61-80%	10	*	18	14	23	7	17	22
81-95%	10	14	21	14	6	7	3	*
>95%	*	29	11	*	6	*	*	*
Weight	(21)	(7)	(27)	(7)	(15)	(15)	(28)	(9)
< 5 %	18	*	4	*	*	*	*	11
5-20%	14	*	7	29	13	13	7	33
21-40%	24	14	4	*	*	*	*	11
41-60%	24	14	15	*	*	27	14	34
61-80%	10	14	15	29	13	13	18	*
81-95%	*	14	22	*	20	27	18	*
>95%	10	44	33	42	54	20	43	11
BMI (kg/m^2)	(21)	(7)	(17)	(7)	(14)	(13)	(13)	(9)
< 5 %	10	*	*	*	*	8	8	11
5-20%	10	*	*	*	*	8	8	33
21-40%	24	*	6	*	*	*	*	*
41-60%	18	14	6	*	*	8	8	34
61-80%	18	14	12	57	29	31	31	11
81-95%	10	29	29	*	7	14	14	11
>95%	10	43	47	43	64	31	31	*
Head Circum	(20)	(6)	(27)	(7)	(19)	(15)	(32)	(9)
< 5 %	60	*	15	*	11	53	56	60
5-20%	10	33	11	14	5	*	*	10
21-40%	20	17	30	29	26	27	16	20
41-60%	10	33	18	14	21	20	12	10
61-80%	*	17	15	29	21	*	*	*
81-95%	*	*	4	14	5	*	16	*
>95%	*	*	7	*	11	*	*	*

All parameters are statistically significant in UF and combined data sets (p<0.05)

In the UF patient population, significant seizures occurred in 19/21 deletion patients, but only in 1/7 UPD, 1/7 ID, 8/15 *UBE3A* mutation and 4/10 Class V patients ($p=9.96X10^{-5}$). Similar findings were observed in the combined data set ($p=8.26X10^{-8}$; Table 3-6). In addition, 90% of Class I patients were hypopigmented compared to

family members ($p=3.33X10^{-8}$). However, less than 25% of Classes II-IV and only 44% of patients in Class V showed evidence of hypopigmentation (Table 3-6).

Table 3-5. Clinical data: means

	Class I	Class II		Class III		Class IV		Class V
	UF	UF	All	UF	All	UF	All	UF
Age to sit[†]	1.3±0.6	0.9±0.3	0.9±0.2	0.7±0.1	0.8±0.3	1.0±0.4	1.0±0.4	1.0±0.4
Age to walk*	4.6±2.1	2.5±0.7	2.6±0.9	2.9±1.2	2.4±1.0	2.8±1.0	2.8±0.9	2.6±0.8
First seizure[‡]	1.9±1.1	4.9±3.4	4.8±3.2	5.5±4.9	3.4±3.5	2.7±1.4	4.4±4.9	1.4±0.9
Birth weight	3.1±0.5	3.8±0.2	3.5±0.5	3.6±0.6	3.5±0.7	3.2±0.5	3.2±0.4	3.6±0.5
Maternal age	27.3±5.2	35.0±7.1	30.2±7.5	29.6±6.6	29.1±6.2	25.9±4.1	25.9±4.1	26.3±3.9
Paternal age	29.8±5.9	35.0±8.4	33.3±7.6	29.6±6.9	29.4±6.4	27.5±3.3	27.5±3.3	33.0±9.1
Gestational age	39.2±1.4	39.0±2.0	39.7±1.1	38.4±1.6	37.5±2.1	39.8±1.4	39.8±1.3	39.7±0.8
Age at last visit	10.9±4.9	9.6±3.4	8.9±6.5	14.8±6.0	10.6±5.8	17.3±10.3	17.3±11.0	12.3±11.7

[†]Significant in UF data only
[‡]Significant in combined data set only
*Significant in UF and combined data sets

Table 3-6. Clinical data: percentages

	Class I	Class II				Class III				Class IV				Class V	
	UF	UF		All		UF		All		UF		All		UF	
Walk by 5 yrs*	50	(20)	100	(7)	96	(25)	86	(7)	94	(18)	93	(15)	97	(35)	100	(7)
Feeding Problems	70	(20)	80	(5)	75	(12)	100	(5)	83	(18)	80	(10)	46	(24)	63	(8)
Simple commands*	53	(17)	100	(4)	91	(11)	100	(7)	100	(13)	100	(13)	100	(13)	100	(5)
Seizures[‡]	100	(21)	43	(7)	45	(31)	29	(7)	59	(27)	53	(15)	73	(48)	60	(10)
Significant Seizures*	90	(21)	14	(7)	12	(26)	14	(7)	17	(18)	53	(15)	41	(41)	40	(10)
Hypopigmented*	90	(21)	17	(6)	20	(20)	0	(7)	14	(22)	15	(13)	23	(43)	44	(9)

*Significant in both data sets
[‡]Significant in UF set only

Discussion

Mutations in *UBE3A*

We have accumulated an AS repository of 104 classical Angelman patients from 93 different families, each of whom has been studied extensively by us at the clinical and molecular levels. Previous mutation analyses by other laboratories revealed a very low *UBE3A* mutation detection rate (5-38%) in sporadic patients with normal DNA methylation at 5' *SNRPN* (Baumer et al., 1999; Fang et al., 1999; Malzac et al., 1998; Moncla et al., 1999a; Russo et al., 2000; van den Ouweland et al., 1999). Five

explanations can account for the paucity of *UBE3A* mutations in these patients: 1. Patients from these studies do not have AS, but instead have disorders that mimic AS; 2. Many *UBE3A* mutations occur in non-coding regions; 3. *UBE3A* can be inactivated by other mechanisms; 4. Other genes in the ubiquitin pathway (genome wide) also cause an AS phenotype; 5. Angelman-associated mutations occur in other 15q11-13 genes.

One goal of our study was to further narrow these possibilities and try to establish whether or not other AS genes existed. Therefore, we restricted our mutation analysis to 20 AS families seen by University of Florida geneticists, eliminating patients with mimicking conditions. We hypothesized that if only one AS gene existed, then 60-80% of our classical AS patients would have intragenic *UBE3A* mutations. We would not expect to find mutations in 100% of our patients because some mutations will invariably lie in non-coding regions of the gene. Overall, we found mutations in 50% of our 20 classical AS patients; 44% of our sporadic cases (7/16) and 75% (3/4) of our familial cases had mutations within *UBE3A*. Although we have found the highest *UBE3A* mutation detection rate in sporadic patients who have AS, the lower than expected frequency suggests that alternative explanations must exist for the lack *UBE3A* mutations in the remaining sporadic Class V patients.

The simplest explanation for the relative lack of mutations in patients with normal DNA methylation at 5' *SNRPN* is that multiple mutations occur outside of the coding region. Although formally a possibility, only two patients have been found with lesions that disrupt either the *UBE3A* gene or an upstream element (Greger et al., 1997; Greger et al., 1994). Furthermore, no mutations have been reported in the 5' UTR of *UBE3A* and Southern analysis of our patients failed to detect genomic rearrangements in our 20 families.

In the 5' region of imprinted genes, the repressed allele is usually methylated, while the expressed allele is unmethylated. In cancer, DNA methylation of the promoter region has been shown to inactivate tumor suppressor genes, while demethylation of promoters can lead to activation of proto-oncogenes (Tycko, 2000). With the exception and *Mash2*, and now *UBE3A*, the promoter regions of imprinted genes show differential methylation based on the parental origin of the chromosome (Glenn et al., 1997; Razin and Cedar, 1994; Tilghman, 1999). In addition, demethylation has been shown to occur in long-term cell culture (LaSalle et al., 1998), reactivating the maternal allele of *SNRPN* (Saitoh and Wada, 2000). Several paternal-only expressed genes within the AS/PWS imprinted domain display parent-of-origin DNA methylation imprints in peripheral blood leukocyte (PBL) DNA (Driscoll et al., 1992; Glenn et al., 1996; Jay et al., 1997; Jong et al., 1999; Kubota et al., 1996a; Sutcliffe et al., 1994). However, when we examined the CpG island at 5' *UBE3A* we found no evidence for a DNA methylation imprint in DNA derived from PBL, brain tissues or germ cells. Both the maternal and paternal alleles were completely unmethylated, even in tissues that had previously revealed imprinted expression of *UBE3A* (Lossie and Driscoll unpublished data; Rougeulle et al., 1997; Vu and Hoffman, 1997).

It is generally accepted that DNA methylation of promoter regions is closely associated with transcriptional repression (Bird and Wolffe, 1999). Although no one is certain how DNA methylation serves to repress transcription, candidates include direct blockage of transcription factors to their binding sites, alteration of local chromatin structure through histone interactions and interplay among methylated DNA, hyperacetylated histones and various DNA binding proteins, such as MeCP2 (Bird and Wolffe, 1999). With the exception of brain, *UBE3A* is expressed biallelically in all

tissues examined (Rougeulle et al., 1997; Vu and Hoffman, 1997). Our data demonstrate that 5' *UBE3A* is normally unmethylated in a variety of tissues. Therefore, we hypothesized that methylation of the maternal CpG island at 5' *UBE3A* could repress transcription of the maternal allele of *UBE3A* in Class V patients. However, Southern analysis revealed that Class V patients are completely unmethylated at 5' *UBE3A* in PBL DNA, indicating that they do not inactivate *UBE3A* by inappropriate methylation. To fully eliminate this possibility brain DNA from Class V patients needs to be examined.

So far, our experiments have suggested that mutations in *UBE3A* are not the sole cause of Angelman syndrome; additional factors must also be involved. The most obvious candidates are other genes in the ubiquitin pathway, an extremely complex cascade that initiates when ubiquitin is activated at its C-terminus by the E1-activating enzyme (Ciechanover et al., 2000; Wilkinson, 2000). Once activated, the ubiquitin molecule is transferred to one of about twelve E2 ubiquitin-conjugating enzymes via a high-energy thiol-ester bond. Next, an E2 enzyme pairs with one of several dozen E3 ubiquitin-protein ligases, forming a complex that transfers activated ubiquitin first to the E3 enzyme then subsequently to the targeted protein. The E4 enzyme then polymerizes the ubiquitin, producing a multi-ubiquitinated protein that is recognized and degraded by the 26S proteosome. This E2-E3 interaction is very specific; UBE3A (an E3 enzyme) will form complexes with the ubiquitin-conjugating enzymes UbcH7 and UbcH8, but not UbcH5 or UbcH6 (Kumar et al., 1997). Furthermore, the temporal and spatial expression patterns of some E3 ligases may add an additional layer of precision targeting (Anan et al., 1998).

Any protein that interacts with UBE3A in this cascade and all proteins that UBE3A targets for degradation are good candidates for patients grouped in Class V. So

far, four proteins have been identified as substrates for UBE3A-mediated degradation: p53, HHR23A, the Src family kinase Blk and UBE3A itself. It is not clear how any of these genes could be involved in the etiology of AS, and given the complexity of the ubiquitin system, identification of the specific AS gene or genes involved is a very daunting task.

Alternatively, other 15q11-13 genes may play important roles in the etiology of Class V patients. *UBE3A* is a complex gene subject to tissue-specific imprinting and alternative splicing (Albrecht et al., 1997; Kishino and Wagstaff, 1998; Rougeulle et al., 1997; Vu and Hoffman, 1997; Yamamoto et al., 1997). In certain cell types in human and mouse brain, *UBE3A* is preferentially expressed from the maternal chromosome. It is not clear from the present data if the human *UBE3A* gene shows global but leaky imprinted expression or demonstrates tightly regulated cell-specific imprinted expression where some cells types are imprinted, while neighboring cells are not. Making the discovery of the mechanism of imprinted expression of *UBE3A* even more difficult is the detection of an antisense transcript that overlaps the last six exons of *UBE3A* (Rougeulle et al., 1998).

The *UBE3A* antisense transcript is a good AS candidate, as it exhibits an expression profile that hauntingly mirrors *UBE3A* imprinted expression, as the paternal-only expressed antisense transcript is only active in the tissues that also show imprinted expression of *UBE3A*. The paternal-only expressed antisense transcript has only been demonstrated to be active in the tissues that also show imprinted expression of *UBE3A* (Rougeulle et al., 1998). One can envision a scenario whereby expression of the antisense transcript from the paternal chromosome specifically inactivates the paternal *UBE3A* allele *in cis* (Brannan and Bartolomei, 1999; Rougeulle et al., 1998). According to this model, if the antisense transcript were activated biallelically in the

brain, both the maternal and paternal alleles of *UBE3A* would be repressed. It would be interesting to determine if Class V patients demonstrated biallelic expression of the *UBE3A* antisense transcript in brain.

Genotype-Phenotype Correlations

The second major goal of this study was to establish phenotypic profiles for each molecular class so physicians could accurately diagnose AS and be able to predict outcomes for the families of patients with Angelman syndrome. Recent studies have shown that UPD (Fridman et al., 2000b; Smith et al., 1997) and ID (Burger et al., 1996; Gillessen-Kaesbach et al., 1999; Saitoh et al., 1999) patients have less severe phenotypes than the deletions. In addition, Moncla and colleagues (1999b) revealed that as a group, non-deletion patients (Classes II-IV) were also less severely affected. However, genotype-phenotype correlations have only revealed differences among the first four classes in head circumference and frequency of seizures (Moncla et al., 1999b). Furthermore, no previous studies have reported on the phenotype associated with Class V patients.

Genotype-phenotype analyses of our patients revealed that all five classes had the four cardinal features of AS: severe developmental delay; profound speech impediment; the AS-specific movement disorder; and the characteristic Angelman behavior. Furthermore, some classes have additional characteristics that are often associated with the Angelman syndrome, such as seizures, microcephaly and hypopigmentation. Out of 22 separate parameters, 16 demonstrated statistical differences among the five classes, at $p<0.05$. Our patients correlate well with those in the literature, with similar findings in 13/16 categories. Our statistical models demonstrated that the five classes could be separated into four phenotypic groups: Class I; Classes II and III; Class IV; and Class V. Multiple clinical distinctions can be

made between the classes, with differences most striking in pigmentation; growth parameters; achievement of developmental milestones; and severity, frequency and age at onset of seizures.

We found that deletion patients as a group have the most classical and severe phenotype. They achieve developmental milestones later and to a lesser degree than the other classes, and are less likely to follow simple commands. Class I patients have the highest incidence of severe seizures (90%) and hypopigmentation (90%) of all five classes. In addition, deletion patients tended to have a complete absence of speech (100%), a normal BMI distribution and severe microcephaly.

In contrast, UPD and ID patients (who have indistinguishable phenotypes) are much less severely affected. They have low incidences of hypopigmentation (≤ 20%), microcephaly and severe seizures (≤ 20%). In addition, about 50% of UPD and ID patients from our repository can speak a few words. Patients in Classes II and III are larger, heavier and often have better coordination. These two groups have very high BMI indices. More than 70% of these individuals are above the 80th percentile for their age group.

Our data correlate well with that of Fridman and colleagues (2000b) who compared 19 AS patients with UPD from multiple studies to 21 deletion patients (Class I). Their analysis showed that Class II patients also displayed a milder phenotype similar to our observations. The Class II patients were heavier, walked earlier and were less likely to have seizures, microcephaly or a complete lack of speech. Analyses of Class III patients from other studies revealed that patients with imprinting mutations also had a milder phenotype. They were less likely to have microcephaly and often had normal pigmentation (Burger et al., 1996; Saitoh et al., 1999). In addition, some studies revealed that patients from Classes II and III had fewer

seizures, early onset obesity and better motor skills (Fridman et al., 2000b; Gillessen-Kaesbach et al., 1999; Smith et al., 1997).

UBE3A mutation patients fall somewhere in the middle. They are statistically similar to deletion patients with 53% of patients having significant or recurring seizures, early onset of seizures in most patients, a complete absence of speech in more than 85% of patients and a high incidence of microcephaly. However, statistically, Class IV patients are similar to UPD and ID patients in the development of motor skills, ability to follow simple commands, pigmentation and development of obesity. Moncla and colleagues (1999b) examined Class IV patients individually for head circumference and seizures. They also concluded that *UBE3A* mutation patients had high incidences of seizures and microcephaly.

Class V patients are very interesting. Although these patients have no detectable chromosome 15 abnormality, statistically they are most similar to the deletion patients. Class V patients have normal height, weight and BMI distributions, similar to that observed in Class I patients. They also typically present with a complete absence of speech, early onset of seizures, hypopigmentation and microcephaly. However, Class V patients walk about two years earlier than deletion patients, and their seizures are less likely to be recurring or as severe as those observed in patients from Class I.

Since Class V patients tend to have a more severe phenotype than that observed in Classes II, III and IV, it is likely that disruption of more than one gene is causative of AS in these patients. Perhaps Class V patients have a mutation in an upstream gene on 15q11-13 that affects not only *UBE3A*, but also additional genes that give rise to a more severe AS phenotype in these individuals. A good candidate gene would be the *UBE3A* antisense transcript. Alternatively, Class V individuals may have

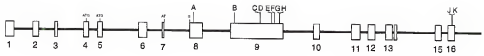
mutations in other genes in the ubiquitination pathway. Since the phenotype is severe, we would predict that the mutation would be in a gene that controlled the degradation of multiple proteins.

Our data indicate that Class I patients comprise a contiguous gene disorder, with haploinsufficiency of multiple genes exacerbating the lack of the maternally expressed *UBE3A* gene. Patients in Classes II and III have the least severe and most atypical phenotype, suggesting that a double dose of the paternal-only expressed genes may be ameliorating the AS phenotype in UPD and ID patients. Alternatively, the moderate phenotype associated with *UBE3A* mutation patients may suggest that haploinsufficiency of *UBE3A* may be detrimental during development and into adulthood. Additionally, *UBE3A* protein levels may be tightly regulated in the central nervous system and peripheral tissues. It is likely that a combination of the double dose of the paternally expressed genes and haploinsufficiency of *UBE3A* both contribute to the differences in phenotype observed between Classes II and III and Class IV patients. Of all four non-deletion classes, the patients with no detectable chromosome 15 abnormality most closely resemble Class I patients.

One puzzling finding is that over 50% of UPD, ID and *UBE3A* mutation patients become extremely obese by puberty. These findings strongly insinuate that the three classes must share a common genetic mechanism for this observation. The UPD and ID patients suggest that a double dose of a paternal gene or genes could cause the obesity. However, this explanation does not account for the *UBE3A* mutation patients. Is it possible that disruption of the maternally-inherited *UBE3A* gene can stabilize a protein that is responsible for the obesity phenotype or activate the maternal allele of a paternally expressed gene?

This integrated study of the differing genotype-phenotype correlations found among all classes of Angelman syndrome patients reveals that distinct clinical subgroups exist. Although mutations in *UBE3A* are sufficient to cause the four cardinal features of Angelman syndrome as well as recurrent seizures and microcephaly, other 15q11-13 genes must contribute to the development of motor skills, severity of seizures, cognition, growth and obesity.

A.



B.

Site	Gene Mutation	Predicted Protein	Inheritance	Patient
A	G648A	C21Y	Maternal	AS179P
B	980 del 2	frameshift	Maternal	AS181P1
B	980 del 2	frameshift	Maternal	AS181P2
C	1522 del 2	frameshift	Maternal	AS180P1
C	1522 del 2	frameshift	Maternal	AS180P2
D	1521 del A	frameshift	*de novo*	AS106P
E	C1835T	R417X	*de novo*	AS178P
F	1930 del 2	frameshift	Maternal	AS141P
G	C2030T	R482X	*de novo*	AS194P
H	A2033T	R483X	Maternal	AS169P1
H	A2033T	R483X	Maternal	AS169P2
H	A2033T	R483X	Maternal	AS169P3
H	A2033T	R483X	Maternal	AS169P4
J	3092 del A	frameshift	*de novo*	AS101P
K	3093 del 4	frameshift	*de novo*	AS152P

Figure 3-1. *UBE3A* mutations in AS patients from the UF repository. A) Genomic organization of *UBE3A*. *UBE3A* spans 120 kb of genomic DNA. Gene is shown to scale. Exons (☐) are numbered numerically from 1-16. Translation (ATG) initiation sites are depicted. The primary translation initiation site begins in exon 8. The location of each mutation is indicated by a letter (A-K). B) Description of mutations. The mutation from each family (A-K) is shown in column 2. Column 3 describes the predicted protein changes, while column 4 shows the inheritance of the mutation.

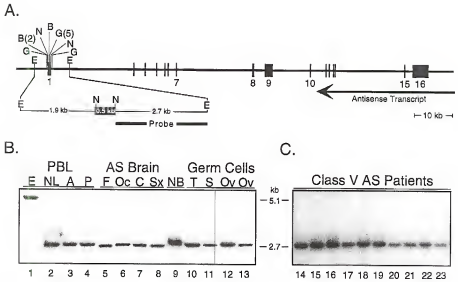


Figure 3-2. DNA methylation analysis of 5' *UBE3A*. **A)** Genomic structure of *UBE3A* and the 5' CpG island (0.5 kb black bar) drawn to scale. Selected restriction enzyme sites are shown: *Eco* RI (E), *Not* I (N), *BssH* II (B), *Eag* I (G). The location of the 2.7 kb Not I-Eco RI probe is shown, as is the position of the 5.1 kb *Eco* RI fragment that surrounds the CpG island. The extent of overlap of the antisense transcript is also shown. If the CpG island is methylated, the 2.7 kb probe will hybridize to a 5.1 kb fragment on genomic DNA digested with *Eco* RI and *Not* I. If the CpG island is unmethylated, the probe with hybridize to itself, revealing a 2.7 kb fragment. **B)** Southern blot of normal (NL), AS (A) and PWS (P) genomic DNA from peripheral blood leukocytes (PBL); frontal cortex (F), occipital lobe (Oc), cerebellum (C) and coronal section (Sx) from AS brain; frontal cortex from normal brain; adult testis (T); sperm (S); and fetal ovary (Ov) digested with *Eco* RI and *Not* I. DNA from normal PBL was also digested with *Eco* RI alone as a control (E). When digested with *Eco* RI and *Not* I, the 2.7 kb probe only hybridized to a 2.7 kb fragment, indicating that the CpG island was unmethylated. **C)** Southern blot of PBL genomic DNA from Class V patients digested with *Eco* RI and *Not* I reveals that the *UBE3A* CpG island is unmethylated in all patients.

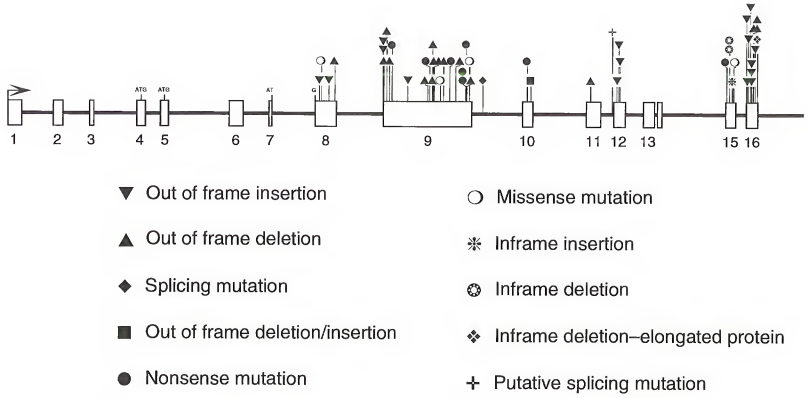


Figure 3-3. Summary of all published mutations in *UBE3A*. Gene is shown to scale. Exons are numbered numerically from 1-16. Transcription (arrow) and translation (ATG) start sites are noted. The primary translation initiation site begins in exon 8. The location of each mutation is indicated by a symbol. Filled symbols indicate protein-truncating mutations. Symbols are stacked at positions where two non-related individuals share a common mutation. There are 51 total mutations in unrelated individuals. Forty-five mutations are unique. Familial mutations are counted once. Most mutations (38/45) are protein truncating and cluster in exons 9 and 16.

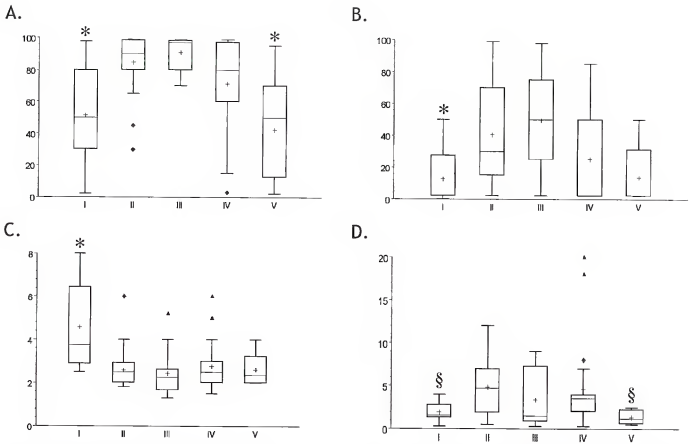


Figure 3-4. Mean BMI, HC, age to walk and onset of seizures. Boxes show the extent of 25-75 % of the patients, while bars (T) depict the range. Median (—), mean (+), outliers (◆) and extreme outliers (▲) are shown. *Statistically significant in UF and combined data sets. §Statistically significant in UF data only. Molecular class (I-V) is shown at the bottom of each graph. The percentiles (A and B) and age in years (C and D) are shown at left. **A)** Body mass index percentiles. **B)** Head circumference percentiles. **C)** Mean age to walk. **D)** Mean age at onset of seizures.

DNA METHYLATION AND EXPRESSION ANALYSES OF *UBE3A* IN THE HUMAN BRAIN

Introduction

The Angelman (AS) and Prader-Willi (PWS) syndromes, two distinct neurobehavioral disorders, are the best-studied examples of genomic imprinting in humans. AS is characterized by severe developmental delay, a profound speech impairment, as well as AS-specific movement and behavioral disorders (Williams et al., 1995b). In stark contrast, patients with PWS initially present with infantile hypotonia and failure to thrive. However, by 18 to 36 months of age, individuals with PWS display several unique characteristics including mild to moderate mental retardation, early onset obesity caused partially by hyperphagia, an obsessive-compulsive disorder, and hypogonadism (Holm et al., 1993). In these very different imprinted disorders, the parental origin of the chromosome 15 abnormality determines the phenotype. PWS occurs from the lack of critical paternally expressed 15q11-13 sequences, while multiple mechanisms that disrupt the maternally inherited E3 ubiquitin protein-ligase gene (*UBE3A*) cause AS.

In the AS/PWS imprinted domain, at least nine known genes and several newly identified imprinted transcripts have been shown to be expressed exclusively from the paternal allele (Dittrich et al., 1993; Driscoll et al., 1992; Glenn et al., 1996; Gray et al., 1999; Jay et al., 1997; Jong et al., 1999; Kubota et al., 1996b; Lee and Wevrick, 2000; Rougeulle et al., 1998; Sutcliffe et al., 1994). These genes include *MKRN3*, *MKRN3-AS*, *MAGEL2*, *NDN*, *SNURF-SNRPN*, *IPW* and the *UBE3A* antisense transcript.

With the exception of *IPW*, all of these genes exhibit imprinted expression in all tissues examined (Lee and Wevrick, 2000). Only the Angelman gene, *UBE3A*, demonstrated preferential expression from the maternal allele, which was limited to the brain (Albrecht et al., 1997; Nakao et al., 1994; Rougeulle et al., 1997; Vu and Hoffman, 1997). Studies in mice revealed that imprinted expression of *Ube3a* was cell-specific, as whole mouse brain failed to show maternal-only transcription (Sutcliffe et al., 1997). Further experiments revealed that complete imprinted expression of *Ube3a* was limited to the CA2 and CA3 regions of the hippocampus, mitral cell layer of the olfactory bulb and purkinje cells in the cerebellum (Albrecht et al., 1997). However, other brain regions demonstrated biased transcription from the maternal allele.

The promoter regions of imprinted genes show differential methylation based on the parental origin of the chromosome (Razin and Cedar, 1994). In the 5' region of imprinted genes, the repressed, maternal alleles are heavily methylated, while the expressed, paternal alleles are undermethylated. DNA methylation of promoter regions is closely associated with transcriptional repression and has been shown to inactivate tumor suppressor genes, while demethylation of promoters can lead to activation of proto-oncogenes (Baylin et al., 1998; Feinberg, 2000; Tycko, 2000). In addition, demethylation has been shown to occur in long-term cell culture, reactivating the maternal alleles of *SNRPN* and *MKRN3* (Saitoh and Wada, 2000). Several paternal-only expressed genes within the AS/PWS imprinted domain display parent-of-origin DNA methylation imprints in peripheral blood leukocyte (PBL) DNA (Boccaccio et al., 1999; Driscoll et al., 1992; Glenn et al., 1996; Jay et al., 1997; Jong et al., 1999; Kubota et al., 1996a; Lee et al., 2000; Sutcliffe et al., 1994). However, when we examined the CpG island at 5' *UBE3A* we found no evidence for a DNA

methylation imprint in DNA derived from PBL, brain tissues or germ cells (Lossie et al., in preparation). Both the maternal and paternal alleles were completely unmethylated, even in tissues that had previously revealed imprinted expression of *UBE3A*.

DNA methylation analyses of the paternally expressed genes suggested that methylation of the promoter regions on the maternal alleles was responsible for imprinted expression. However, no DNA methylation imprint has been shown to exist at *UBE3A*. Therefore, we wanted to test whether specific cell types in the human brain demonstrated imprinted expression of *UBE3A* and determine if some of these imprinted tissues also demonstrated DNA methylation imprints at the CpG island located in the promoter of *UBE3A*.

Materials and Methods

Human Subjects and Tissue Samples

All patients with AS and PWS were deletions with a classical phenotype (Holm et al., 1993; Williams et al., 1995a). Tissues from patients with AS were obtained from the University of Florida Brain Bank, while tissue samples from the PWS and control subjects were obtained from the University of Miami Brain Bank (1889, 1199, 2144 and 2803). These studies were approved by the institutional review board at the University of Florida and informed consent was obtained from all patients and/or parents.

RNA Isolation

Formalin Fixed Paraffin Embedded Samples (FFPE). On day one, tissue sections were deparaffinized in subsequent 15 min. xylene washes of 5 and 2 ml at 37°C. Each wash was followed by centrifugation at 10,500 g for 15 min and removal of

supernatant. Xylenes were removed by two 100% ethanol washes (5 and 2 ml) at 37°C for 30 min. each. A 30 min. centrifugation step at 10,500 g followed each ethanol wash. Pellets were dried briefly before the addition of 2 ml RNA lysis buffer (0.2 M Tris-HCl, pH 7.5, 0.2 M NaCl, 1.5 mM $MgCl_2$ and 2% SDS) and 100 µl Proteinase K (10 mg/ml). Samples were homogenized with a tissumizer, and incubated at 42°C overnight. Another 0.5 ml of RNA lysis buffer and 100 µl of Proteinase K were added each day for 3 successive days. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitated. Genomic DNA was removed by DNase I (Roche) digestion. Five µg of total nucleic acids were incubated at 37°C with 100 U of DNAse I for 60 min. in 1X PCR buffer (Roche), purified by phenol/chloroform extraction and ethanol precipitation.

Fresh Frozen Tissues. RNA was isolated from fresh frozen tissues according to established protocols (Chomczynski and Sacchi, 1987).

RT-PCR

cDNAs (total, *UBE3A* and *UBE3A* antisense) were generated from 250 ng of fresh frozen and FFPE total RNA according to standard procedures (Life Technologies) using either random hexamers or previously published primers (Rougeulle et al., 1998). PCR products from *UBE3A*, the *UBE3A* antisense transcript, *SNURF-SNRPN* and the transferrin receptor (*TR*) were amplified, size fractionated by gel electrophoresis, blotted and hybridized with one of the primers used for PCR. PCR primers from exons 13 and 14 and exon 16 were used to amplify *UBE3A* and the *UBE3A* antisense transcript, respectively (Lossie et al., in preparation). *SNURF-SNRPN* primers from exons 1-3 (previously -1 to 1) have been previously published (Glenn et al., 1996). Primers were designed to amplify a 299 bp product from the *TR*, to control for transcript levels. The forward primer, which starts at nucleotide 346, is 5'-GCTACTTG

GGCTATTGTAAAGGG-3', while the reverse primer is 5'-ACGCCAGACTTTGCTGAGTT-3' and ends at nucleotide 644 of the *TR*. One primer from each PCR reaction was labeled by kinasing (Promega) 400 ng in a standard reaction containing 40 μCi ^{32}P γdATP (Amersham). Labeled primers were hybridized to Southern blots of PCR products overnight at T_H in 6 X SSCP, 0.1% SDS, 1 X Denhardt's and 20 mg/ml single stranded salmon sperm DNA. T_H is T_m minus 5°C. the oligo in 2 X SSCP; 0.1% SDS. Excess probe was removed by two 15 min. washes in preheated, freshly prepared hybridization buffer. Products were visualized by autoradiography.

DNA Methylation

Methylation Specific PCR. Sodium bisulfite treatment of genomic DNA from fresh frozen tissues and subsequent methylation analysis of *SNRPN* was done according to previous protocols (Kubota et al., 1997; Lossie and Driscoll, 1999). Two sets of primers were designed to assess the methylation status of both *Not* I sites in the *UBE3A* CpG island. At the proximal end of the CpG island (more 5') the primers are as follows: methylated primer set (M1F 5'-CGTCGCGGTCGCGAGATT-3' and M1R 5'-ACGCGCCGAATCGACAAAA), unmethylated primer set (U1F 5'-GTGTTGTGGTTGTGAGAT TTGTG-3' and U1R 5'-ACAAACACCCACACTAACAAATAACT-3'). The primer sets that amplify the distal CpG island are as follows: methylated primer set (M2F 5'-GATAGGTA GCGGCGGTTGGCGACGAA-3' and M2R 5'-GCGCCTAAACTACGACGACCGCCTCA-3', unmethylated primer set (U2F 5'-GAATGTTGGGATTTTGGTGGT-3' and U2R 5'-AAACTACAACAACCACCTCACTAATCA-3'). PCR products were stained with ethidium bromide and visualized on an Eagle Eye II photodocumentation system (Stratagene).

Southern Analysis. Southern analysis of *SNRPN* and *UBE3A* were performed according to published protocols (Glenn et al., 1993b; Lossie et al., in preparation).

Results

UBE3A Does Not Demonstrate a DNA Methylation Imprint

In our previous analysis of the different classes of Angelman syndrome patients, we demonstrated that *UBE3A* was unmethylated in all tissues examined, including four distinct regions of the human brain (Figure 3-2). Interestingly, one region, frontal cortex, had previously been shown to demonstrate imprinted expression of *UBE3A*. We developed a novel PCR based assay to assess the DNA methylation status of *UBE3A* by sodium bisulfite.

We designed four sets primers to amplify the proximal and distal regions of the *UBE3A* CpG island (Figure 4-1A). At both the proximal and distal ends of the CpG island, PCR products were only generated from the unmethylated primers in all AS, PWS and control samples (Figure 4-1B). A PCR product from the methylated primers was amplified in cosmid DNA treated with SssI CpG methylase (Figure 4-1B). As a control, the same brain tissues were assayed for methylation status at 5' *SNRPN* (Figure 4-1C). In the AS brain tissues, only the unmethylated *SNRPN* allele was amplified, while only the methylated allele was amplified in PWS brain samples. Both alleles were present in control samples.

Expression Analysis of *UBE3A* and the Antisense Transcript in the Brain

To date, the only region of the human brain proven to show imprinted expression of either *UBE3A* or the antisense transcript is the frontal cortex (Rougeulle et al., 1997). However, mouse studies suggest that other distinct regions of the human brain may also demonstrate imprinted expression of *UBE3A* (Albrecht et al., 1997). We examined imprinted expression of *UBE3A*, the antisense transcript and *SNURF-SNRPN* in brain tissues from AS, PWS and control individuals.

We assessed imprinted expression of *UBE3A* and the antisense transcript by strand-specific RT-PCR (Figure 4-2A). Three cDNA pools were generated from each 250 ng total RNA sample: total cDNA derived from random hexamers, *UBE3A* sense and *UBE3A* antisense transcripts. *SNRPN* and the transferrin receptor were amplified from the total cDNA pool. PCR primers from exons 13 and 14 were used to amplify the *UBE3A* gene, while primers from introns 12 and 14 amplified the *UBE3A* antisense transcript. *UBE3A* was predominantly expressed in PWS brain samples (maternal expression), and the *UBE3A* antisense transcript was predominantly expressed from the paternal allele, as it was observed in AS, but not PWS brains (Figure 4-2B). Prolonged exposure of the *UBE3A* Southern revealed that *UBE3A* was expressed at very low levels in all three AS brain samples. However, *SNRPN* was only expressed from the AS samples. *UBE3A*, the antisense transcript, *SNRPN* and the *TR* were expressed in cDNA pools from control brains (data not shown).

Discussion

DNA methylation of promoter regions is closely associated with transcriptional repression (Bird and Wolffe, 1999). With the exception of *Mash2*, and now *UBE3A*, all well-studied imprinted genes display parent-of-origin methylation imprints (Glenn et al., 1997; Razin and Cedar, 1994; Tilghman, 1999), including all of the well-characterized paternally expressed genes within 15q11-13. The maternal alleles of *MKRN3* (Driscoll et al., 1992), *NDN* (Jay et al., 1997) and *SNURF-SNRPN* (Glenn et al., 1996; Sutcliffe et al., 1994) demonstrate preferentially methylation in the promoter regions. In addition, several anonymous CpG sites within the AS/PWS imprinted domain also demonstrate DNA methylation imprints. The best studied of these is PW71 (Dittrich et al., 1993), but several sites within the imprinting center (IC) also demonstrate DNA methylation imprints (Farber et al., 1999). Including *SNURF-SNRPN*,

ten sites within the IC show differential methylation between the parental chromosomes. Nine of these CpGs are preferentially methylated on the maternal allele.

DNA methylation of the promoter regions of the paternally expressed genes within the AS/PWS imprinted domain correlates well with what is presently known about methylation-induced repression (Bird and Wolffe, 1999). Data from the present study suggest that imprinted expression of *UBE3A* occurs via a novel mechanism. One possible reason for the lack of a DNA methylation imprint in *UBE3A* could have been that differential methylation only occurred in discreet regions of the human brain. However, we would expect that the regions demonstrating imprinted expressed would also reveal a DNA methylation imprint. It is clear from our present studies that no DNA methylation imprint exists at the CpG island at 5' *UBE3A* in six different brain regions, although some of these tissues demonstrate imprinted expression of *UBE3A*. Therefore, it is unlikely that repression of *UBE3A* occurs by DNA methylation at the promoter.

An alternative explanation for imprinted expression of *UBE3A* stemmed from the discovery of a brain-specific *UBE3A* antisense transcript (Rougeulle et al., 1998). Examination of frontal cortex from single AS and PWS samples revealed that *UBE3A* was preferentially expressed from the maternal allele, while the antisense transcript was preferentially expressed from the paternal allele. However, amplification of the 3' UTR revealed biallelic expression of *UBE3A* using the same AS sample (Lee and Wevrick, 2000). Since strand-specific reverse transcription was not done, biallelic expression was expected in this reaction. It is important to note that both transcripts are expressed at low levels (5-10%) from the repressed alleles (Rougeulle et al., 1998). The authors postulated that co-expression of *UBE3A* and the antisense

transcript from the same chromosome would result in silencing of the *UBE3A* gene in *cis*, accounting for the maternal-only expression of *UBE3A* in brain.

Given that *UBE3A* was completely unmethylated in all tissues examined, this was an attractive hypothesis. In order to test this possibility, we analyzed imprinted expression of *UBE3A* and the antisense transcript in two AS patients (paternal only), two PWS individuals (maternal only) and two normal controls (biparental). Our studies supported this model, revealing that *UBE3A* was preferentially expressed from the maternal allele in several regions of the human brain including frontal cortex, hippocampus and cerebellum while the antisense transcript was only expressed from the paternal allele.

The lack of a DNA methylation imprint at 5' *UBE3A* combined with the discordant expression of the *UBE3A* sense and antisense transcripts suggest that DNA methylation of the promoter region of *UBE3A* does not account for imprinted expression. Our data support an alternative mechanism of imprinted expression of *UBE3A*. We believe that expression of the antisense transcript results in repression of *UBE3A*, *in cis*. This could occur through multiple mechanisms, which depend upon nuclear localization of the antisense transcript. If the antisense transcript remains localized to the expressed allele, in a scenario similar to *Xist*, then RNA interference would be a good candidate for repression of *UBE3A*. However, if the antisense transcript can diffuse throughout the nucleus (e. g. act in *trans*), an alternative mechanism would have to account for the allele-specific repression. Perhaps expression of the antisense transcript can cause changes in chromatin structure that precludes expression of *UBE3A*.

Further studies examining DNA methylation and imprinted expression in additional regions of the human brain will help us to better understand the mechanism

of imprinted expression of *UBE3A*, and demonstrate which regions in the human brain are important in the AS phenotype.

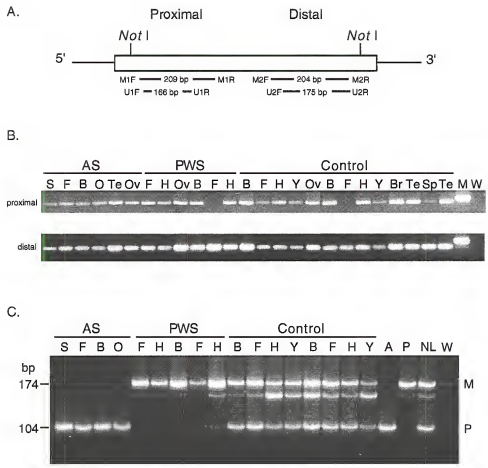


Figure 4-1. Methylation analysis of brain DNA. A. Scale Map of *UBE3A.* exon 1. The proximal and distal regions assayed as well as *Not* I sites are indicated. Primer designations (methylated = M and unmethylated = U) are located below their positions and amplification product sizes are shown. B. Methylation specific PCR at UBE3A. PCR products from the proximal and distal regions are indicated. Brain regions and germ cells are denoted: Coronal section (S), Frontal cortex (F), Cerebellum (B), Occipital lobe (O), Hippocampus (H), Hypothalamus (Y), Brain (Br), Testis (Te), Ovary (Ov) and Sperm (Sp). Cosmid DNA treated with Sss I methylase (M) and water (W) controls are indicated. C. Methylation specific PCR at 5' SNRPN. The maternal, methylated (M) and paternal, unmethylated (P) alleles are shown. Sizes (bp) of each fragment are also indicated. AS, PWS and Control brain regions are the same as above. Control PBL DNAs are also noted: AS (A), PWS (P) and control (NL).

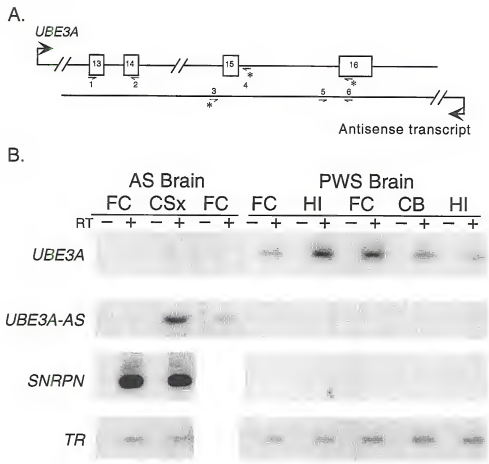


Figure 4-2. Imprinted expression of *UBE3A* in brain. A. Map of *UBE3A* and the antisense transcript. Relevent exons are numbered. Primers used for RT reactions are designated (*). Remaining primers were used for PCR. B. RT-PCR analysis. Regions of the brain are noted: frontal cortex (FC), Coronal section (CSx), Hippocampus (Hi) and Cerebellum (CB). Primers from exons 13 and 14 were used to amplify *UBE3A*. Primers from intron 15 and exon 16 were used to amplify the antisense transcript. Exons 1-3 of *SNRPN* were analyzed. Strand-specific primers were used to amplify *UBE3A* (4 and 6) and the antisense transcript (3). The transferrin receptor (*TR*) was used as a control.

CONCLUSIONS AND DISCUSSION

The past six years have seen very exciting developments occur in Angelman syndrome research. Since early 1997, the field has progressed at a very fast pace. During the past four years, the AS gene (*UBE3A*) has been cloned (Kishino et al., 1997; Matsuura et al., 1997). Within seven months of the report of mutations in *UBE3A* in several AS patients, imprinted expression of *UBE3A* was demonstrated in human and mouse brain (Albrecht et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997). Angelman syndrome was the first human disease shown to be caused by mutations in the ubiquitin pathway, and has paved the way for the cloning of additional disease genes, including the discovery of mutations in another E3 ligase in the non-agouti-lethal a^{18H} mouse (Perry et al., 1998). This mouse displays the *itchy* phenotype, characterized in part by constant itching due to hyperplasia of the forestomach epithelia, and revealed that the ubiquitin pathway was critical for immune response. Several new genes and transcripts have bseen localized to the AS/PWS domain (Lee and Wevrick, 2000), including an antisense transcript was found to overlap the 3' end of *UBE3A* (Rougeulle et al., 1998). Five mouse models of AS have also been generated to help in the study of the mechanisms of imprinted expression of *UBE3A* (Cattanach et al., 1997; DeLorey et al., 1998; Gabriel et al., 1998; Jiang et al., 1998; Johnson et al., 1995). Analyses of AS and PWS patients have demonstrated that the imprinting center (IC) consists of separate AS and PWS domains, although the mechanisms of imprinted expression of the maternal and paternal genes has yet to be discovered.

In addition to these discoveries, my research has revealed several new findings. During the study of one of our AS deletion patients, we discovered that female AS patients have the ability to reproduce (Lossie and Driscoll, 1999). This important discovery has serious implications for families and caretakers of AS patients, and implies that social cognitive behaviors, not physiological defects, prevent most AS patients from reproduction. Studies of the different molecular classes of AS have revealed multiple new findings. I have shown that there are five molecular classes of AS, but four phenotypic AS groups (Lossie et al., in preparation). This research has also revealed that the fifth class of AS patients has a more severe phenotype than the UPD, ID or *UBE3A* mutation patients, implying that multiple genes may be disrupted in these patients. I have examined several regions of the brain, but found no evidence of a DNA methylation imprint associated with *UBE3A*. Out of all of the ≥ 35 known imprinted genes, *Mash2* is the only other known transcript without a DNA methylation imprint (Tilghman, 1999). In addition, my RT-PCR analyses of *UBE3A* and the antisense transcript suggest that imprinted expression of *UBE3A* is regulated *in cis* by the antisense transcript, suggesting that a novel mechanism underlies imprinted expression of *UBE3A* in the brain.

Molecular Classes of AS

Results of my analysis of AS patients from the University of Florida repository demonstrates that five major molecular mechanisms cause AS: Class I) 4 Mb maternally inherited deletions of 15q11-13; Class II) paternal uniparental disomies (UPD); Class III) imprinting defects (ID); Class IV) patients with intragenic mutations in *UBE3A*; and Class V) patients with no known defects.

Class I Patients

The advent of high resolution chromosome analysis in the late 1980s allowed the localization of the AS gene to human chromosome 15 by the detection of 3-5 Mb interstitial deletions of 15q11-q13 in several AS patients (Kaplan et al., 1987; Magenis et al., 1987). Initial observances indicated that the AS deletion was identical to that seen in Prader-Willi syndrome. However, evidence soon indicated that in Class I AS patients, the deletion was always maternally inherited (Knoll et al., 1989; Zackowski et al., 1993), while PWS deletions were always of paternal origin (Butler et al., 1986; Knoll et al., 1989).

Deletion patients have the most severe phenotype and represent a contiguous gene disorder, with haploinsufficiency of several non-imprinted genes along 15q11-13 exacerbating the phenotype. Some striking differences between Class I individuals and the other patients are the frequency and severity of seizures. All Class I patients have seizures, and 90% have recurring bouts of epilepsy. The most obvious genes affecting seizures in deletion patients are the three *GABA* receptors, which are located about 2 Mb distal to *UBE3A*. Mice with null mutations in *Gabaβ3* recapitulate several of the AS characteristics, including balance abnormalities, seizures, abnormal EEG patterns, sleep disturbances and learning defects (DeLorey et al., 1998). However, mice heterozygous for the disruption have wild type features. It would be interesting to study the phenotype of mice with disruptions in all three *GABA* receptors. In addition to seizures, most deletion patients are hypopigmented, microcephalic and have poorly developed motor skills.

Class II Patients

Uniparental disomy of chromosome 15 also occurs in both syndromes-maternal UPD in PWS (Class II; (Mascari et al., 1992; Nicholls et al., 1989) and paternal UPD in

AS patients (Class II; (Malcolm et al., 1991; Nicholls et al., 1992). The identification of AS patients with UPD of chromosome 15 conclusively demonstrated that AS was an imprinted disorder, as absence of the maternally derived 15q11-13 chromosomal region resulted in the Angelman syndrome phenotype.

In the UF patient repository, 7 % of patients had paternal UPD for chromosome 15 (Lossie et al., in preparation). Hypopigmentation only occurred in 20% of UPD patients, and less than 15% of UPD patients suffered from recurring episodes of epilepsy or microcephaly. In addition, 96% of Class II patients could walk by five years of age, twice the frequency observed in deletion patients. These patients had a much less severe phenotype than the deletion patients, indicating that twofold expression of the paternal-only genes may be ameliorating the AS phenotype.

Class III Patients

A third subset of AS and PWS patients (ID patients) have mutations in the mechanism(s) involved in the imprint process. Mutations and spontaneous defects in the imprinting center (IC) cause the maternal chromosome to adopt a paternal epigenotype along 15q11-13, with these individuals having paternal DNA methylation and expression imprints (Glenn et al., 1993a; Reis et al., 1994). About 50% of Class III AS patients have detectable microdeletions in the imprinting center. The remaining Class III AS patients have no detectable mutations, but show an AS epigenotype across the 2 Mb imprinted domain (Buiting et al., 1998). Recently the AS smallest region of overlap (SRO) for the IC region has been narrowed to 0.9 kb, while the PWS-SRO in the IC has been narrowed to 4.3 kb (Buiting et al., 1999).

Although they a different molecular mechanism causes AS in IC and UPD patients, statistically the two classes have indistinguishable phenotypes. This

observation demonstrates that the only imprinted domain on chromosome 15 is the AS/PWS region.

Class IV Patients

Several patients have biparental inheritance of chromosome 15 with normal DNA methylation at several loci within the AS/PWS imprinted domain. Mutation analysis of *UBE3A*, revealed protein truncating mutations in several AS individuals (Kishino et al., 1997; Matsuura et al., 1997). Mutation analysis of UF patients revealed the existence of five molecular classes, as only 44% of sporadic patients with normal methylation had intragenic mutations within *UBE3A* (Lossie et al., in preparation). Mutations, which were mostly protein truncating, were found throughout the coding region, with clusters in the HECT domain and in exon 9.

As a group, *UBE3A* mutation patients achieve developmental milestones faster, are more likely to follow simple commands, have less severe seizures that begin at a later age and are heavier than deletion patients. However, unlike UPD and ID patients, individuals from Class IV tend to have a higher incidence of significant or recurring seizures and often display microcephaly. Moncla and colleagues (1999b) examined Class IV patients individually for head circumference and seizures. They also concluded that *UBE3A* mutation patients had high incidences of seizures and microcephaly.

Class V Patients

Previous studies showed that the majority of patients with normal DNA methylation do not have detectable abnormalities on chromosome 15 (Fang et al., 1999; Malzac et al., 1998). In UF patients, about 10 % of all classical AS patients fall into this group. Not much is known about the etiology of AS in these patients. It has

been postulated that they have mutations in other genes in the ubiquitin pathway or in *UBE3A* substrates. Alternatively, they could have disruptions in other 15q11-13 genes that interfere with *UBE3A* expression.

Class V patients are very interesting. They were not significantly different from deletion patients across several phenotypic categories. Class V patients have normal height, weight and BMI distributions, similar to that observed in Class I patients. They also typically present with a complete absence of speech, early onset of seizures, hypopigmentation and microcephaly. However, Class V patients walk about two years earlier than deletion patients, and their seizures are less likely to be recurring or as severe as those observed in patients from Class I. Since Class V patients tend to have a more severe phenotype than that observed in Classes II, III and IV, it is likely that disruption of more than one gene is causative of AS in these patients. That they closely resemble UPD, ID and *UBE3A* mutation patients in their type and severity of seizures indicates that *UBE3A* may also be disrupted in these patients. Class V patients also had a higher frequency of hypopigmentation compared with Classes II, III and IV. Maybe these phenotypes can yield clues as to the mechanism(s) involved in the pathogenesis of AS in these individuals.

Genotype-phenotype Correlations among the Five Classes of AS

Several articles in recent literature have compared the phenotypes associated with patients from Classes II and III with Class I individuals (Burger et al., 1996; Fridman et al., 2000b; Gillessen-Kaesbach et al., 1999; Saitoh et al., 1997; Saitoh et al., 1999; Trent et al., 1997). However, extensive genotype/phenotype correlations among the first four classes of AS patients has been limited (Moncla et al., 1999b), and no studies have examined the phenotype associated with Class V patients.

Genotype/phenotype analyses of our patients revealed that all 5 classes of patients had the four cardinal features of AS: severe developmental delay and/or mental retardation; severe speech impediment; an unsteady, ataxic gait with uplifted hands; and the characteristic happy affect. Furthermore, some Classes have additional characteristics that are often associated with the Angelman syndrome, such as seizures, microcephaly and hypopigmentation. Our statistical models demonstrated that distinct phenotypic differences occur amongst the molecular classes. The five classes of patients can be separated into four phenotypic groups: Class I, Classes II and III, Class IV, and Class V. Overall, Class I patients are the most severely affected, while Classes II and III are the least. Classes IV and V differ from each other at several criteria, but fall between Class I and Classes II and III in severity. Multiple clinical distinctions can be made between the classes, with differences most striking in pigmentation; growth parameters; achievement of developmental milestones; and severity, frequency and age at onset of seizures.

Our data indicate that Class I patients comprise a contiguous gene disorder, with haploinsufficiency of multiple genes exacerbating the lack of the maternally expressed *UBE3A* gene. Patients in Classes II and III have the least severe and most atypical phenotype, suggesting that a double dose of the paternal-only expressed genes may be ameliorating the AS phenotype in UPD and ID patients. Alternatively, the more severe phenotype associated with *UBE3A* mutation patients could indicate that haploinsufficiency of *UBE3A* may be detrimental during development and into adulthood. Additionally, *UBE3A* protein levels may be tightly regulated in the central nervous system and peripheral tissues. It is likely that a combination of the double dose of the paternally expressed genes and haploinsufficiency of *UBE3A* both

contribute to the differences in phenotype observed between Classes II and III and Class IV patients.

Of all four non-deletion classes, the patients with no detectable chromosome 15 abnormality most closely resemble Class I patients. Perhaps Class V patients have a mutation in an upstream gene on 15q11-13 that affects not only *UBE3A*, but also additional genes that give rise to a more severe AS phenotype in these individuals. A good candidate gene would be the *UBE3A* antisense transcript. Alternatively, Class V individuals may have mutations in other genes in the ubiquitination pathway. Since the phenotype is severe, we would predict that the mutation would be in a gene that controlled the degradation of multiple proteins.

What is the Epigenetic "Mark?"

Imprinted genes display several distinguishing characteristics: differential methylation and allele-specific expression patterns between the maternal and paternal alleles, the presence of non-coding and/or antisense RNA transcripts, asynchronous DNA replication timing, GC rich sequences, and direct repeats (Glenn et al., 1997; Razin and Cedar, 1994; Sleutels et al., 2000). In addition, genomic imprinting can impart transcriptional changes in a single gene or alter expression patterns of groups of genes across several megabases (Mb; Glenn et al., 1997). Recent attention has focused on determining the mechanism that establishes the epigenetic mark, or imprint, between the maternal and paternal alleles. Multiple mechanisms have been proposed to perform this tagging, including DNA methylation, chromatin structure, *cis*-acting elements and DNA replication timing. Human and murine studies indicate that the mechanisms involved in imprinting must be able to: 1.) Set the imprint in the gametes; 2.) Maintain this epigenetic modification in somatic tissues; 3.) Alter the expression or repression of imprinted genes and 4.) Erase the imprint

during gametogenesis and reset it according to the sex of the offspring (Driscoll, 1994).

DNA Methylation

DNA methylation is a good candidate for the establishment and maintenance of the imprint. After replication, hemi-methylated DNA is recognized by the maintenance DNA methyltransferase protein, which methylates the daughter strand, propagating the methylation status of the allele. Driscoll et al., (1992) were the first to propose DNA methylation as a diagnostic tool for identifying imprinted genes. With the exception of *UBE3A* and *Mash2*, all imprinted genes display differential methylation sites that show parent-of-origin methylation imprints (Glenn et al., 1997; Razin and Cedar, 1994; Tilghman, 1999). Targeted disruption of the *DNA methyltransferase* gene (*Dnmt1*) in mice provided initial evidence for the role of DNA methylation in genomic imprinting (Li et al., 1993). Inactivation of *Dnmt1* repressed the normally active *Igf2r*, *Igf2* and *Kvlqt1* alleles and activated the repressed copies of the *H19*, *Xist*, *Cdk1c* and *Snrpn* genes (Caspary et al., 1998; Li et al., 1993; Shemer et al., 1997). In order for DNA methylation to be accepted as an "imprinting mark," one must identify imprints that are established in the germ line and maintained somatically during and after development. In addition, these tags should escape the global wave of demethylation that occurs during early embryogenesis (Brandeis et al., 1993; Szabo and Mann, 1995; Tada et al., 1997). To date, gametic imprints have been identified in five genes: *SNRPN* (human and mouse), *MKRN3*, *Igf2r*, *H19* and *Gnas* (Glenn et al., 1996; Jones et al., 1996; Jong et al., 1999; Liu et al., 2000; Shemer et al., 1997; Stöger et al., 1993; Tremblay et al., 1995). My studies demonstrated that DNA methylation was not responsible for imprinted expression of *UBE3A*, as no methylation imprint was found in any tissue (Lossie et al., in preparation).

Cis-acting Elements

In addition to primary DNA methylation imprints, other *cis*-acting elements can be found in multiple imprinted regions. These elements often have long-range effects, and differential DNA methylation appears to be a common factor in these *cis*-acting elements. For example, within the 2 Mb imprinted domain on human chromosome 15q11-13, the Prader-Willi syndrome imprinting center (PWS-IC) controls the establishment and maintenance of imprinted expression of the paternally expressed genes along the entire imprinted domain (Bielinska et al., 2000). Additional studies from this region indicate that there is also a separate Angelman syndrome imprinting center (AS-IC) that potentially works in the same manner on the homologous chromosome (Buiting et al., 1995; Farber et al., 1999; Saitoh et al., 1996). Similar imprinting centers have also been identified in the *Igf2/H19* imprinted region on mouse chromosome 7C (Birger et al., 1999; Hark et al., 2000; Khosla et al., 1999; Sasaki et al., 2000; Szabo et al., 2000). It will be interesting to determine if patients with mutations in the PWS IC (PWS ID patients) or AS IC (AS ID patients) express the *UBE3A* antisense transcript.

Chromatin Structure

Although it is evident that DNA methylation plays an important role in the establishment of the primary imprint, other factors are probably in the allele-specific repression. Several experiments have demonstrated a significant correlation between DNA methylation at CpG islands and transcriptional silencing (Ng and Bird, 1999). Experiments using the herpes simplex virus thymidine kinase (*HSV TK*) gene gave the initial clues that DNA methylation could be tied to chromatin structure (Buschhausen et al., 1987). When naked DNA (methylated or unmethylated) was microinjected into rodent cells, it took over eight hours for *HSV TK* to be silenced in cells containing

methylated DNA. However, when *in vitro* assembled chromatin was introduced, *HSV TK* was inactive. Recent studies have been able to intimately connect DNA methylation with chromatin structure (Ng and Bird, 1999). The discovery that the CpG binding protein, MeCP2, bound DNA in a methylation-dependent mechanism and inhibited transcription gave the first clues that the two processes were linked (Nan et al., 1997; Nan et al., 1996). Subsequent experiments by several groups revealed that the transcriptional repression domain of MeCP2 recruits Sin3A and the histone deacetylase complex, inducing histone deacetylation and transcriptional silencing (Ng and Bird, 1999).

Identification of a gene that shows differential chromatin structure between the maternal and paternal alleles in the context of DNA methylation and expression imprints would provide good evidence of a connection between DNA methylation, chromatin structure and imprinted expression. *In vivo* chromatin studies revealed that the unmethylated expressed alleles of *Snrpn*, *H19* and *U2af1-rs1* were hypersensitive to *in vivo* nuclease digestion, insinuating that a DNA methylation imprint was linked to an open chromatin conformation (Feil et al., 1995; Hark and Tilghman, 1998; Khosla et al., 1999; Schweizer et al., 1999; Shibata et al., 1996; Szabo et al., 1998). In addition, analysis of histone acetylation by chromatin immunoprecipitation demonstrates that the inactive alleles of *Igf2* and *H19* (maternal and paternal, respectively) are enriched for hypoacetylated histones, a hallmark of repressed genes (Pedone et al., 1999).

DNA Replication Timing

Another potential candidate for the imprinting signal is DNA replication timing. The maternal and paternal homologues of most genes replicate synchronously during S phase of the cell cycle (Glenn et al., 1997). In contrast, the homologous gene regions

of imprinted domains have been shown to replicate asynchronously, with the paternal allele usually replicating earlier than the maternal homologue (Argyriou-Tirita et al., 1996; Izumikawa et al., 1991; Kitsberg et al., 1993; Knoll et al., 1994; LaSalle and Lalande, 1995; Lin et al., 1995; Simon et al., 1999; Smrzka et al., 1995; White et al., 1996; Wutz et al., 1998). Further analysis of the imprinted 15q11-13 domain has shown a homologous association between the maternal and paternal homologues in normal lymphoblast cell lines (LaSalle and Lalande, 1995). However, asynchronous DNA replication timing does not appear to correlate with changes in imprinting of *IGF2* or *H19* in patients with Beckwith-Wiedemann syndrome (Squire et al., 2000). In addition, asynchronously replicating domains often extend past the imprinting clusters (Glenn et al., 1997).

Models for Imprinted Expression

In an intriguing model brought upon by studies of the mouse *Aprt* locus, Turcker has postulated that CpG islands themselves are targets for *de novo* methylation, probably by a *Dnmt3* or *Dnmt3a* methyltransferase (Turker, 1999). After the initial methylation at discreet foci, regional methylation of a CpG island would spread bidirectionally through maintenance methylation, a replication-dependent process requiring *Dnmt1*. The methylation status of the CpG island would remain in flux; methylation would compete with demethylation to produce a stable methylation pattern. Other recent experiments have led (Antequera and Bird, 1999) to propose a mechanism for why CpG islands are often found at gene promoters. They believe that CpG islands serve dual roles, acting both as origins of replication and as promoters for genes that are active at totipotent stages of development. If these models from non-imprinted loci prove true in imprinted regions, then DNA methylation could be the

epigenetic mark of imprinted genes, linking DNA replication, chromatin structure, cis-acting elements and imprinted expression.

Several models have been proposed for the function of the IC. The first model proposed that the paternal component of the IC (PWS-IC) controlled the maternal to paternal epigenotype switch, while the maternal component of the IC (AS-IC) regulated the paternal to maternal switch (Dittrich et al., 1996). However, this model requires activation of the maternal allele during gametogenesis. Although no maternal expression has been detected in fetal brain or lung, fetal oocytes and primordial germ cells have not been examined (Buiting et al., 1999). It is possible that the IC contains germ cell-specific transcripts that are maternal in origin.

In an alternative explanation, the PWS-IC regulates expression of the paternally expressed genes in 15q11-13, while the AS-IC represses activation of the PWS-IC (Figure 1-4B; Brannan and Bartolomei, 1999). On the paternal chromosome, the PWS-IC activates transcription of the paternally expressed genes along the 2 Mb imprinted domain, which includes the *UBE3A* antisense transcript (Figure 1-4A, B). Patients with mutations in the PWS-IC would fail to activate paternal gene expression, causing PWS. In oogenesis and somatic tissue, the AS-IC represses the PWS-IC, silencing the paternally expressed genes. AS patients with imprinting defects would fail to inactivate the PWS-IC, causing biallelic expression of the paternal-only genes. One prediction of this model would be that *UBE3A* would only demonstrate imprinted expression in tissues where the *UBE3A* antisense transcript was concurrently expressed. In support of this model, the *UBE3A* antisense transcript is only expressed in brain, the one tissue that demonstrates imprinted expression of *UBE3A* (Rougeulle et al., 1998; Rougeulle et al., 1997). In addition, recent experiments demonstrated that *UBE3A* was preferentially expressed from the maternal allele, while the antisense

transcript was preferentially expressed from the paternal allele in several regions of the human brain (Lossie et al., in preparation). A second prediction of this model is that an IC deletion on the maternal chromosome that extended from the AS-IC through the PWS-IC would not give an AS phenotype because the paternally expressed genes would not become activated, due to a lack of a PWS-IC. In agreement with this model, none of the AS-IC deletion patients deletes the PWS-IC.

My preliminary experiments suggest that the antisense transcript plays a role in the regulation of imprinted expression of *UBE3A*. One possible mechanism for this regulation would be through RNA interference due to double stranded RNA interactions. In order for this mechanism to apply, the antisense transcript must not be able to diffuse throughout the nucleus. If it could, then both the maternal and paternal alleles of *UBE3A* would be repressed. If the *UBE3A* antisense transcript does diffuse throughout the nucleus, then changes in higher order nuclear structure could be a mechanism for imprinted expression of *UBE3A*. It is possible that the nuclear localization of *UBE3A* is different in brain than other tissues. This localization could set up epigenetic differences in chromatin structure on the maternal and paternal chromosomes, provide different microenvironments for distribution of transcription factors and ultimately result in preferential expression from the maternal allele in brain.

Interestingly, *PWCR1*, a small nucleolar (sno) RNA has been mapped within 1 Mb of *UBE3A* (de Los Santos et al., 2000). Like most of the genes within the AS/PWS domain, *PWCR1* is preferentially expressed from the paternal allele and is expressed at high levels in brain. Within a 50 kb stretch at the *PWCR1* locus, 25 copies of genes with high homology to *PWCR1* are found. It is possible that the presence of these and/or other noncoding RNAs are responsible for establishing precise nuclear

architecture. This would not be surprising, since noncoding RNAs and antisense transcripts appear to play a major roll in epigenetic gene silencing. For example, the noncoding *Tsix* transcript, which is also antisense to *Xist*, has been shown to be involved in the epigenetic regulation of X-inactivation (Lee et al., 1999a). In addition, the roX RNAs have been shown to facilitate the male specific hypertranscription on the paternal X chromosome in *Drosophila* (Kelley et al., 1999).

Future Directions

My studies suggest that mutations in *UBE3A* are not the sole cause of Angelman syndrome; additional factors must also be involved. The next logical step in better understanding the etiology of AS is to identify all of the substrates that UBE3A targets for degradation. Any protein that interacts with UBE3A in this cascade and all proteins that UBE3A targets for degradation are good candidates for patients grouped in Class V. So far, four proteins have been identified as substrates for UBE3A-mediated degradation: p53, HHR23A, the Src family kinase Blk and UBE3A itself. Perhaps an even more intriguing future exploration is to determine if other 15q11-13 genes may play important roles in the etiology of Class V patients. Possible candidates are the *UBE3A* antisense transcript and the sense transcript located downstream of *UBE3A*. These genes then need to be tested for their possible involvement in AS. It is not clear how any of these genes could be involved in the etiology of AS, and given the complexity of the ubiquitin system, identification of the specific AS gene or genes involved is a very daunting task.

In addition to determining the etiology of AS in Class V patients, my recent studies on imprinted expression of *UBE3A* and the antisense transcript suggest that imprinted expression of *UBE3A* is complicated. To better understand the biology, the

next logical step would be to examine more regions of the brain to try to establish the tissue-specificity of imprinted expression of *UBE3A* and the antisense transcript. I think my research indicates that a comprehensive *in situ* analysis of all regions of the brain would greatly help to answer whether or not the antisense transcript can regulate imprinted expression of *UBE3A*.

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BIOGRAPHICAL SKETCH

Amy Lossie was born on August 15, 1967, in Kalamazoo, Michigan. Growing up on Three Mile Lake in nearby Paw Paw, Amy's early childhood was spent enjoying the outdoors where she developed an intense curiosity of nature and love of water. In high school, Amy was diagnosed with a rare birth defect. This was a major turning point in Amy's life. Luckily, her friends and family were completely supportive of her during this hard time. She relied heavily on her best friend, Andy Glomski, who has to this day remained her staunch ally. During her extensive hospital stay, Amy was exposed to medical research for the first time, and she developed an intense desire to learn what caused her birth defect.

Amy entered The University of Michigan in 1985. She lived a full life in college. In addition to her studies in cellular and molecular biology, she found plenty of time to have fun. She spent football Saturdays at the Big House cheering on the Wolverines and found a job working in a molecular pharmacology lab. Amy realized that she loved the bench more than books. Along with her lab mate, Joan Taylor, Amy spent many evenings and weekends finishing experiments. The friends she made during this time in her life greatly influenced her career choices. Through example, Joan showed Amy that she could follow her dream and become an academic scientist. After graduating with a BS in 1990, Amy left pharmacology and entered the field of genetics. Working with Dr. Sally Camper, Amy learned the fundamentals of Genetics research. She worked hard and earned the respect of Dr. Camper, who gave Amy the

opportunity to design her own experiments and write her own papers. In Sally's lab, Amy learned that it took blood, sweat and total devotion to be a successful graduate student.

In the summer of 1995, Amy entered Dr. Dan Driscoll's laboratory as a graduate student in the Center for Mammalian Genetics. She has spent the past five and a half years researching genomic imprinting in the Angelman syndrome. Amy grew scientifically under the direction of Dr. Driscoll, who was her mentor in every meaning of the word. Dr. Driscoll funneled valuable resources into Amy's research, enabling her to successfully complete her studies. Amy will never forget that devotion.

Amy intends to earn her Ph. D. from the University of Florida in the fall of 2000. Upon completion of her dissertation, Amy will continue her scientific training in the laboratory of Dr. Monica Justice at Baylor College of Medicine. Amy plans to study developmental genetics using the mouse as a model system. Her research will focus on characterization of one mutation of an allele series of *Odz4*. This interesting mutation may also be subject to genomic imprinting. Amy intends to use her knowledge of imprinting to further the understanding of *Odz4* in the mouse. Her long term goals are to develop a strong academic research laboratory in human and mouse genetics, where she will educate and train young scientists.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel J. Driscoll, Chair
Associate Professor of Pediatric Genetics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Camilynn I. Brannan
Assistant Professor of Molecular Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Margaret R. Wallace
Associate Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas P. Yang
Professor of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 2000

Dean, College of Medicine

Dean, Graduate School